REMARKS

The Office Action mailed April 2, 2004, set a three-month shortened statutory period for response expiring July 2, 2004. The period for response is extended three months to October 2, 2004, pursuant to the petition for extension of time under 37 CFR 1.136(a) submitted herewith. This amendment is therefore timely filed.

Before this amendment, claims 1-32 and 35-39 were in the application. Claims 33 and 34 were cancelled in the Amendment filed February 19, 2002. Claim 39 was added on December 18, 2004.

Non-elected claims 6-32 and 35-38 are cancelled herein without prejudice to the prosecution thereof in a continuing application.

Claim 1 has been amended to change the transitional phrase from "comprising" to "consisting essentially of," thereby more particularly defining Applicants' preferred polypeptides. Applicants reserve the right to prosecute any deleted subject matter in a continuation application.

Claims 1 and 3 have been amended to delete the non-elected subject matter. Applicants reserve the right to prosecute the cancelled subject matter in a continuing application.

Claims 2 and 39 have been cancelled as being essentially redundant to amended claim 1.

Claim 3 has been amended to place this claim in independent form.

New claim 40 is drawn to compositions comprising SEQ ID NO:6, the elected species, and a pharmaceutically acceptable vehicle. Support for this amendment can be found in the specification, for example, on page 16, lines 7 to 11.

The application, as amended, contains claims 1, 3-5 and 40.

REJECTION UNDER 35 U.S.C. § 101

Claims 1-5 and 39 (only claims 1 and 3-5 of which remain in the application) are rejected under 35 U.S.C. § 101 on the grounds that the invention is not supported by a specific asserted utility, a well established utility, or a substantial utility for the reasons given in prior office actions. With respect to Applicants' previous response, the Examiner contends that a) it has not been demonstrated that it is the SEQ ID NO:6 protein isoform which accumulates in various tumor cells and is differentially expressed in a number of cancers compared to normal control, and b) no nexus has been established between the p73 of Tominaga et al and the claimed SR-p70, SEQ ID NO:6, since a review of Tominaga did not reveal that the construct provided by D. Caput is the p73 disclosed in WO 99/66946, and given the known splice variants of p73, no nexus has been established between p73 of Tominaga et al. and the p73 of WO 99/66946 of El-Deiry.

This rejection is traversed and reconsideration and withdrawal thereof is respectfully requested for the reasons given hereinbelow.

In order to find that an asserted utility is not specific or substantial, the burden is on the Examiner to make a *prima facie* showing that it is more likely than not that a person of ordinary skill in the art would not consider *any* utility asserted by Applicants to be specific or substantial. The Examiner must also provide evidence sufficient to show that each statement of asserted utility would be considered false by a person or ordinary skill in the art. Applicants submit that the Examiner has failed to meet this burden.

Applicants have set forth in the specification numerous specific, substantial and credible utilities. For example, the disclosure provides that a polypeptide comprising an amino acid sequence consisting of SEQ ID NO: 6 can be used in therapeutic and diagnostic applications related to the phenomenon of apoptosis and/or cell transformation of tumors. The instant disclosure additionally provides that antibodies to p73 (Applicant's SR-p70) are useful in detecting an abnormal accumulation of p73 proteins in biological samples, which makes them useful for detecting cancers or monitoring the progression or remission of pre-existing cancers (specification p 14, line 13, p. 15, line 2). Alternatively, the protein itself can be used to detect auto-antibodies against p73 in patients' sera (specification p. 15, lines 14-17), which has been confirmed by others in the relevant art. These utilities are both specific and substantial, and, in view of the following, would more than likely be considered credible by those of ordinary skill in the art.

First, the accumulation of p73 in tumor cells and the testing for serum antibodies thereto has been documented in the literature by Tominaga et al. (*Br. J. Cancer*, 2001, 5:84(1):57-63; hereinafter Tominaga). According to Tominaga, p73 protein does indeed accumulate in tumour cells (p. 57, right column.), p73 can be used (and hence, SR-p70) to detect the presence of p73 antibodies in the sera of patients with different types of cancer (as was acknowledged by the Examiner, see Paper 25, page 12), and p73 (SR-p70) can be used to monitor the follow-up of patients during therapy (Figure 6). To establish a nexus between the p73 of Tominaga and SR-p70, or specifically to SEQ ID NO:6, applicants provided the Examiner with a comparison of SR-p70, which corresponds to SEQ ID NO:6, with the sequence of p73 set forth in WO 99/66946, which the Examiner acknowledged are residue by residue identical. Thus, applicants were able to establish that what is now referred to as "p73," is in fact the SR-p70 described in the instant invention.

However, the Examiner contends that given the known splice variants of p-73, no nexus has been provided between the p73 useful for detecting cancer-related autoantibodies of Tominaga and the instantly claimed invention. Applicants respectfully disagree. Not only was the p73 of Tominaga derived from the constructs obtained from D. Caput, one of the inventors in the instant application, (Tominaga, Acknowledgements, page 62), but the Examiner has failed to provide *any* evidence that a splice variant was used other than the full length p73 variant. In fact, Figure 1 of Tominaga makes it clear that "p73" is a polypeptide of 636 amino acids, and thus, would necessarily be the p-73 full length variant, which is commonly referred to as p-73α (*see* Figure 9, Ikawa et al. *Cell Death and Differentiation* (1999) 6, 1154). Since the Examiner previously recognized in the Office Action dated June 18, 2003, (page 12) that SEQ ID NO:6, also referred to as SR-p70a, is the p-73 alpha splice variant, then the requisite "nexus" between the p73 of Tominaga and SR-p70a, SEQ ID NO:6 has clearly been established. Therefore, given that there is no reason to believe that p73 and SR-p70 are not identical, Applicants have provided the requisite nexus and do in fact teach a credible utility for the claimed invention.

Additionally, Applicants submit that p73 is differentially expressed in a number of cancers as compared to normal tissue (Ikawa et al p. 1157, right-hand column), and, even if p73 is not differentially expressed in all possible tumor types, the instant application does provide a means of detecting those tumors in which p73 is differentially expressed. The Examiner maintains, however, that applicants have not proven that p73 is differentially expressed since no nexus has been provided that it is the SEQ ID NO:6 protein isoform which accumulates in various tumor cells and is

differentially expressed in a number of cancers to normal. Although applicants disagree, provided herewith are additional publications confirming that p73α (Applicants' SEQ ID NO:6) is differentially expressed in a number of cancer cells. Specifically, Puig et al. have also confirmed a statistically significant association between p73α expression and tumor stage and tumor category in bladder cancer (*Clinical Cancer Research*, 9, 5642, Nov. 15, 2003), and Frasca et al. have shown that transcriptionally active (full length) p73α is differentially expressed in tumor as compared to normal cells (*Cancer Research*, 63, 5829, Sept. 15, 2003). Frasca et al. additionally observed that transcriptionally active p73 expression was elevated in tissue specimens derived from malignant tumors of the thyroid epithelium, but was undetectable in normal thyroid tissue (p. 5831).

Moreover, to provide yet additional evidence of the utility of the present invention, Applicants point to United States Patent No. 6,451,979 issued to Kaelin et al. (hereinafter "Kaelin"), reaffirming that p-73α (referred to as NBS-1α) suppresses tumor growth and induces apoptosis. Kaelin's NBS-1α polypeptide of SEQ ID NO:3 is again residue for residue identical to Applicants' SEQ ID No:6, except for a typographical error in Kaelin's disclosure at residue number 601 of the NBS-1α (p73α) protein (see Figure 1A; see also Figure 1C, remedying the error). Since NBS-1α and SEQ ID NO:6 are identical, a "nexus" has been established, thus supporting the utility of SEQ ID NO:6 in suppressing tumor growth and inducing apoptosis. Moreover, Kaelin's patented claims cover an isolated monoclonal antibody that specifically binds to SEQ ID NO:3 (hence, Applicants' SEQ ID NO:6). It would follow that if an antibody that specifically binds to this polypeptide (which antibodies are also described in the instant specification on pages 13 to 14) has a credible utility, then there must be a recognizable utility for the polypeptide (applicants SEQ ID NO:6) itself.

In view of the above-remarks, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1-5 and 39 under 35 U.S.C. § 101.

REJECTIONS UNDER 35 U.S.C. § 112

Claims 1-5 and 39 (only claims 1 and 3-5 of which remain in the application) are rejected under 35 U.S.C. § 112, first paragraph, for reasons set forth in the paper mailed June 18, 2003, section 3, page 7, which was based on the reasons set forth in Paper No. 15, Sections 7 and 8, pages 4-5 and previously, which was for the reasons set forth in Paper No. 12, Section 15, pages 11, and 12-14; essentially for the following reasons: 1) since the claimed invention is not supported by a specific utility, a well-established utility, and a substantial utility, than one skilled in the art would

not know how to use the claimed invention; 2) while being enabled for a composition comprising SEQ ID NO:6, the specification does not reasonably provide enablement for polypeptides comprising a biologically active fragment of SEQ ID NO:6 derived from SEQ ID NO:6; and 3) while being enabled for a composition comprising SEQ ID NO:6, the specification does not reasonably provide enablement for a polypeptide comprising a sequence selected from SEQ ID NO:6 (see Paper No. 12, page 12), since the claims encompass polypeptides comprising non-disclosed amino acid sequences attached to the polypeptide of SEQ ID NO:6, that it would be expected that a substantial number of polypeptides encompassed by the claims would not share either structural or functional properties with the polypeptide of SEQ ID NO:6, and that the specification fails to tell how to make or use the polypeptides, and fails to provide any working examples to provide guidelines to one skilled in the art.

This rejection is traversed and reconsideration and withdrawal thereof is respectfully requested for the reasons provided hereinbelow.

Initially, the portion of this rejection under 35 U.S.C. §112, first paragraph, for lack of enablement due to an alleged lack of utility is believed overcome in view of the foregoing arguments which clearly establish that applicants do indeed teach a utility for the claimed invention.

Insofar as this rejection pertains to polypeptides comprising a biologically active fragment of SEQ ID NO:6 derived from SEQ ID NO:6, it is believed that this basis for rejection was overcome by the amendments to claim 1 filed January 30, 2002, wherein the references to sequences derived from SEQ ID NO's: 2, 4, 6, 8, 10, 13, 15, 17, and 19 were deleted to more particularly define Applicants' preferred polypeptides.

With respect to the instant rejection based on the alleged non-enablement for a polypeptide comprising a sequence selected from SEQ ID NO:6, this rejection is believed overcome by the above-described amendment to claim 1 wherein the transitional phrase "comprising" has been replaced with "consisting essentially of."

Moreover, it is respectfully requested that new claim 40 be entered as it is drawn to a composition comprising SEQ ID NO:6 and a pharmaceutically acceptable vehicle, which the examiner acknowledged was enabled by the specification (see Paper No. 12, page 12, line 3-4, and page 14, lines 12-13).

Thus, in view of the above-described amendments and remarks, the rejection of claims 1-5 and 39 based on 35 U.S.C. § 112, first paragraph, is believed overcome, and reconsideration and withdrawal thereof is respectfully requested.

Claims 1-5 and 39 (only claims 1 and 3-5 of which remain in the application) stand rejected under 35 U.S.C. § 112, first paragraph, for the reasons stated in the paper mailed June 18, 2003, section 3, page 7; and specifically on the grounds that with a 73% overall dissimilarity of p53 and with 41% dissimilarity in the putative DNA binding region, the function of the SEQ ID NO:6 polypeptide could not be predicted, and that given the ubiquitous expression of p73 alpha and the generally low expression in both normal and tumor tissues, it is unclear how one would use SEQ ID NO:6 in treating the phenomenon of carcinogenesis. The Examiner further contends that it cannot be predicted, as Applicants have done, whether this splice variant is expressed *in vivo*, whether this splice variant is differentially expressed, whether this splice variant is involved in carcinogenesis or whether it can be used in methods drawn to the prophylactic, diagnostic or therapeutic methods as suggested by Applicants. However, the Examiner does acknowledge that Tominaga teaches the detection of p73 autoantibodies in patients with various types of cancer. Nevertheless, it is urged that Tominaga does not teach which splice variant of SR-p70 the autoantibodies are specific for, or whether SR-p70 is expressed *in vivo* and concludes that no evidence has been provided which would allow one of skill in the art to predict that the invention will function as contemplated, and that the specification provides insufficient guidance on how to use the claimed invention with a reasonable expectation of success.

This rejection is traversed and reconsideration and withdrawal thereof is respectfully requested for the reasons given hereinbelow.

The instant specification describes SR-p70 (SEQ ID NO. 6), which is identical to p73; teaches the manner of making it (specification page 11, line 6, to page 13, line 22) and teaches how to use it (specification p. 15, lines 14-27), which use is confirmed by Tominaga. Moreover, the instant invention provide a means of detecting tumors in which p73α is differentially expressed, such

differential expression of p73α being confirmed by Frasca et al. and Puig et al. Accordingly, the instant specification fully meets the requirements of 35 U.S.C. § 112.

Nevertheless, the Examiner continues to urge that given the known splice variants of p73, no nexus has been provided between the p73 useful for detecting cancer-related autoantibodies of Tominaga and the instantly claimed invention. However, in view of Applicants above-remarks responding to the rejection under 35 U.S.C. § 101, and in particular, the numerous observations substantiating such a nexus, including the fact that the p73 protein described by Tominaga contained 636 amino acids (illustrated in Figure 1), the p73 protein of Tominaga is, therefore, the p73α full length variant which corresponds to SEQ ID NO:6.

Thus, the instant specification describes the invention, teaches the manner of making the invention, and teaches how to use the invention, which is all that is required under 35 U.S.C. § 112, first paragraph. Therefore, reconsideration and withdrawal of the rejection under 35 U.S.C. § 112, first paragraph, is requested.

Copies of Puig et al. (*Clinical Cancer Research*, 9, 5642, Nov. 15, 2003), Frasca et al. (*Cancer Research*, 63, 5829, Sept. 15, 2003) and U.S. Patent No. 6,451,979 (Kaelin et al.) referred to hereinabove are submitted herewith for the convenience of the Examiner.

There being no remaining issues, this application is believed in condition for favorable reconsideration and early allowance, and such actions are earnestly solicited.

Respectfully submitted,

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p73 Tumor-Suppressor Activity Is Impaired in Human Thyroid Cancer[1]

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ABSTRACT

The p73 protein is a member of the p53 family and, like p53, can induce cell-cycle arrest and apoptosis in response to DNA damage. Because the loss of p53 function is responsible for the progression of well-differentiated thyroid cancer to more aggressive phenotypes, we hypothesized that p73 might also be involved in thyroid carcinogenesis.

We find that normal thyrocites do not express p73, whereas most thyroid malignancies are positive for p73 expression. However, the p73 protein of thyroid cancer cells is unresponsive to DNA-damaging agents, failing to elicit a block of the cell cycle or an apoptotic response. Notably, overexpression of transcriptionally active p73 in thyroid cancer lines can arrest the cell cycle but is still unable to induce cell death. The loss of p73 biological activity in neoplastic thyroid cells is partly explained by its interaction with transcriptionally inactive variants of p73 (ΔNp73) and with mutant p53. Our findings suggest that the functional impairment of p73 could be involved in the development of thyroid malignancies, defining p73 as a potential therapeutic target for thyroid cancer.

INTRODUCTION

Malignancies of the thyroid follicular epithelium are regarded as a unique model to study human carcinogenesis, because they comprise tumors with different clinical and histological features (1). Indeed, papillary and follicular thyroid cancers are slow-growing, well-differentiated tumors, whereas anaplastic thyroid cancers are undifferentiated neoplasias that behave much more aggressively, usually leading to the death of the patient <1 year from diagnosis (2, 3). Well-differentiated thyroid cancers maintain some morphological features of the normal thyroid and are often responsive to radioiodine treatment, because they usually retain the expression of the sodium iodide symporter (4). On the contrary, anaplastic tumors have lost any significant resemblance to the structural organization of a normal thyroid follicle, and are unresponsive to both radioactive iodine treatment and chemotherapeutic agents (5).

From a molecular standpoint, well-differentiated thyroid cancers are often initiated by genetic events that involve the improper activation of cellular proto-oncogenes (6). In up to 40% of papillary thyroid carcinomas, rearrangements of the *RET* or *NTRK1* tyrosine kinases with a plethora of activating genes represent the first event in tumor development (7, 8). In ~35% of follicular thyroid carcinomas, neoplastic transformation is triggered by activating mutations of the *RAS* gene or by the fusion of the *PAX8* transcription factor with the gene encoding for peroxisome proliferator-activated receptor γ (1, 9). Unfortunately, whereas some of the genetic events responsible for the development of well-differentiated thyroid cancers have been identified, the molecular mechanisms that generate the lethal anaplastic thyroid cancer are still unclear (10). To date, the only established genetic alteration involved in the dedifferentiation process leading to anaplastic thyroid tumor development is the loss of the *p53* tumor suppressor gene (11).

p73 belongs to a family of proteins defined by the *p53* tumor suppressor gene (12, 13). p53 and p73 share significant homologies in their structural organization characterized by an NH_2-terminal transactivation domain, a central DNA-binding domain, and a COOH-terminal oligomerization domain (14). In addition, both p53 and p73 can block the cell cycle or induce cell death in response to DNA damage, or after the activation of selected oncogenes (15, 16).

Despite these characteristics, p53 and p73 can play distinct and sometimes opposing biological roles in cancer development (17). Unlike p53, p73 can be expressed as a wide variety of significantly different transcripts. Abnormal splicing mechanisms generate p73 mRNAs defective of the second exon (Δ2p73), or both the second and third exons (Δ2–3p73; Refs. 18–20). Moreover, the presence of two functional promoters can alternatively produce TAp73[4] (Ref. 4; transcribed from the first exon) or ΔNp73 (transcribed from a previously silent 3′ exon) isoforms (21). Both the aberrant splicing of the first exons and the activation of an alternative promoter generate p73 transcripts that lack a functional transactivation domain (22). Because these ΔTAp73 isoforms are still able to oligomerize with transcriptionally competent p73 variants, they behave as dominant-negative proteins that interfere with the activity of TAp73 and can cause malignant transformation (16, 23). This composite picture is additionally complicated by the discovery that each NH_2-terminal p73 variant can present multiple COOH terminus ends originated by the alternative splicing of the last five exons of p73 (24–26). In the end, the net biological function of p73 results from the multiple interactions of transcriptionally active (TAp73) and transcriptionally silent (Δ2p73, Δ2–3p73, and ΔNp73) isoforms of the protein (14, 16).

Another intricate issue concerns the direct interplay between p53 and p73. Multiple reports have shown that ΔNp73 can interfere with wild-type p53 activity by competing for the p53 binding sites on several promoters (20, 27–29). At the same time, it has been demonstrated that certain p53 mutants can bind TAp73, thereby inactivating its transcriptional activity (30, 31).

Here we report that TAp73 and ΔNp73 are expressed in well-differentiated and undifferentiated thyroid carcinomas but are not present in normal thyroid tissues, suggesting that p73 might be involved in thyroid carcinogenesis. Indeed, in thyroid carcinomas, TAp73 is not up-regulated by DNA-damaging agents and fails to induce either cell-cycle arrest or apoptosis.

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[4] The abbreviations used are: TAp73, transcriptionally active p73; ΔNp73, p73 isoforms lacking the NH_2-terminus (first three exons); ΔTAp73, p73 isoforms lacking a functional transactivation domain; GFP, green fluorescent protein; PI, propidium iodide; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR; FACS, fluorescence-activated cell sorter; HA, hemagglutinin.

MATERIALS AND METHODS

Cells. Human thyroid cancer cell lines TPC-1 (papillary), BC-PAP (papillary), NPA (papillary), WRO (follicular), ARO (anaplastic), and KAT-4 (anaplastic), provided by Alfredo Fusco and Massimo Santoro (University of Naples Federico II, Italy); ARO Neo, ARO IF, and ARO C5 a gift of Alfredo Pontecorvi (Catholic University of the Sacred Heart of Jesus, Rome, Italy); SW-1736 (anaplastic), Hth-74 (anaplastic), and C-643 (anaplastic) provided by Nils Heldin (Uppsala University, Uppsala, Sweden); FF-1 (anaplastic) established in our laboratory; ONCO-DG-1 (papillary) and 8505-C (papillary) purchased from DMSZ (Braunschweig, Germany); and FTC-133 (follicular), FTC-238 (follicular,) and 8305-C (anaplastic) purchased from ECACC (Salisbury, United Kingdom) were grown in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 2 mM glutamine, 10% FBS, and 100 μg/ml penicillin and streptomycin. Normal thyroid cells in primary culture were obtained from surgical specimens after treatment with 1 mg/ml collagenase IV (Sigma). The human osteosarcoma cell line Saos-2 and simian kidney cell line COS-1 were provided by Jean Wang (University of California, San Diego, CA) and cultured in DMEM (Sigma) additioned with 10% FBS, and 100 μg/ml penicillin and streptomycin. The human hepatocarcinoma cell line HepG2 (American Type Culture Collection, Manassas, VA) was grown in MEM supplemented with FBS and antibiotics as described above.

Human Thyroid Tissue Samples. Human thyroid cancer specimens were obtained at surgery and stored in liquid nitrogen until processing.

Immunohistochemistry. Immunohistochemical staining was performed on 7-μm thick sections of unfixed tissue specimens. Sections were cut with a cryostat at −30°C, fixed with acetone at −20°C for 10 min, and hydrated with PBS, at room temperature for 45 min. After blocking in 2% normal serum for 20 min, sections were incubated overnight with the anti-p73 polyclonal antibody Ab-6 (1:100; NeoMarkers, Labvision Corp., Fremont, CA). Specific labeling was detected with biotin-conjugated antimouse/antirabbit IgG and avidin-biotin peroxidase complex. Sections were counterstained with hematoxylin QS, examined, and photographed using an Olympus BH-2 microscope.

Immunofluorescence. Cells were fixed in 3.7% formaldehyde, permeabilized with PBS/0.3% Triton X-100, blocked with PBS/10% normal goat serum, and incubated with primary antibodies for 1 h. To detect endogenous TAp73 we used monoclonal antibody 429 (Imgenex, San Diego, CA), whereas HA-tagged p73 was revealed by an anti-HA monoclonal antibody (CRP, Berkley, CA). The cells were then incubated with Alexa-conjugated (Alexa Fluor 594 or 488) secondary antibodies (Molecular Probes, Leiden, the Netherlands) for 1 h. To visualize the cytoplasm, the cells were also incubated with Alexa-conjugated phalloidin (Molecular Probes) for an additional 30 min. The cells were finally counterstained with Hoechst (Sigma) to color the nuclei. Epifluorescence microscopy was performed with an Olympus microscope. The images were digitally acquired with an Orca CCD Camera (Hamamatsu, Hamamatsu City, Japan) and processed with the Image-Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD).

Immunoprecipitation and Immunoblot Analysis. Cell lysates were prepared in radioimmunoprecipitation assay buffer containing 0.1% SDS and protease inhibitor mixture (Roche Biochemical Inc, Basel, Switzerland). For immunoprecipitation (IP) experiments, 1 mg of cell lysate was incubated for 2 h with 2 μg of antibody. After an incubation with protein A-Sepharose (Amersham Biosciences, Uppsala, Sweden), samples were resuspended in loading buffer, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% milk-Tris Buffered Saline with 0.5% Tween and then immunoblotted with primary antibodies (1 mg/ml). Appropriate horseradish peroxidase-conjugated secondary antibodies were added at 1:2000 (Amersham Biosciences), and proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

The following antibodies were used for immunoprecipitation: mixture of monoclonal antibodies against different epitopes of p73 Ab-4 (NeoMarkers), polyclonal anti-HA antibody (CRP), polyclonal anti-ΔNp73 antibody (Oncogene Research, La Jolla, CA), monoclonal antibody DO-1 against the NH_2 terminus of p53 (Santa Cruz Biotechnology, Santa Cruz, CA), and monoclonal anti-GFP antibody (CRP).

The following antibodies were used for Western blotting: monoclonal antibodies 429 and 1288 (Imgenex), respectively, recognizing the transactivation domain and the DNA-binding domain of p73; polyclonal Ab-5 antibody (NeoMarkers) recognizing the DNA binding domain of p73; monoclonal DO-1 antibody (Santa Cruz Biotechnology); polyclonal anti p21 antibody (Santa Cruz Biotechnology); monoclonal anti-Bax antibody (Trevigen Inc., Gaithersburg, MD); monoclonal anti-β actin antibody (Sigma); and monoclonal anti-HA and anti-GFP antibodies (CRP). The proteasome inhibitor MG132 was purchased from Sigma.

Transcript Analysis by RT-PCR. Total RNA (1 μg) was reverse transcribed with Superscript II (Invitrogen, Paisley, United Kingdom) and oligodeoxythymidylic acid primers. Two μl of the synthesized cDNA were then combined in a PCR reaction specific for TAp73, using primers 5′ CGGGACGGACGCCGATG 3′ (forward) and 5′ CTTGGCGATCTGGCAGTAG 3′ (reverse) spanning exons 1 and 5 of the *p73* gene (fragment size, 542 bp). The ΔNp73 transcript was detected using primers 5′GCTGTACGTCGGTGACCC3′ (forward) and 5′ CTTGGCGATCTGGCAGTAG 3′ (reverse; fragment size, 322 bp). Transcription of the housekeeping *ELE1* gene was carried out using primers 5′ ATTGAAGAAATTGCAGGCTC 3′ (forward) and 5′ TGGAGAAGAGGAGCTGTATCT 3′ (reverse). All of the TAp73 transcripts expressed by thyroid cancer lines included in this report were screened for possible mutations by automatic sequencing of RT-PCR amplicons generated using primers 5′ CGGGACGGACGCCGATG 3′ (forward) and 5′ CAGATGGTCATGCGGTACTG 3′ (reverse). To assess the p53 status of all thyroid cancer cells studied, the p53 coding sequence was amplified by RT-PCR using primers 5′ CCTTCCGGGTCACTGC 3′ (forward) and 5′ TGGGCCTTGAAGTTAGAGAAA 3′ (reverse). PCR products were then subjected to automatic sequencing (BMR-CRIBI Sequencing Service, University of Padua, Padua, Italy).

Plasmids and Transfections. pCDNA3.0-HA-TAp73α, pCDNA3.0-HA-ΔNp73α, pCDNA3.1-p53, and pBOS-H2B-GFP were provided by Jean Wang (University of California, San Diego, CA). pCDNA3.1-p53-GFP was a gift of Geoffrey Wahl and Jane Stommel (The Salk Institute, LA Jolla, CA); p21Luc and BaxLuc were donated by Giovanni Blandino (Regina Elena Cancer Institute, Rome, Italy). pCDNA3.0-HA-GFP-TAp73α was prepared in our laboratory. The GFP cDNA was obtained by RT-PCR using pEGFP (Clontech, Palo Alto, CA) as a template. The primers used were 5′ GCTAGCATGGTGAGCAAGGGCGAG 3′ (forward) and 5′ GATGCTAGCCTTGTACAGCTCGTCCAT 3′ (reverse). The GFP cDNA was inserted into pCDNA3.0-HA-TAp73α at the *Nhe*I site, located between the HA tag and the TAp73α sequence. All of the transfections were performed in six-well plates with Fugene 6 (Roche Biochemical Inc.) according to the manufacturer's instructions (DNA:fugene ratio 1:3), and cells were processed 24 h after transfection. To obtain stable transfected clones, cells were plated onto 100-mm Petri dishes and grown in complete medium containing 1 mg/ml of Geneticin (Invitrogen). After 2 weeks, clones were isolated, grown in 35-mm Petri dishes, and screened by Western blot for HA-GFP-TAp73α expression.

Luciferase Assay. The p21Luc or BaxLuc constructs were cotransfected with pCDNA3.1, pCDNA3.0-HAp73, or pCDNA3.1-p53 (DNA ratio 1:1). A vector coding for the Renilla luciferase (provided by Enrico Conte, University of Catania, Catania, Italy) was also cotransfected in all of the conditions (DNA ratio 1:20). Twenty-four h after transfection, the cells were processed with the Dual Luciferase Assay (Promega Corp., Madison, WI) according to the manufacturer's instructions. Luciferase activity was normalized for transfection efficiency (Renilla activity).

Cell Cycle and Apoptosis Evaluation. Cells were synchronized for 36 h in serum-free medium without leucine and released in complete medium for 12 (cell cycle) or 72 h (apoptosis) in the presence or the absence of 2 μM doxorubicin. Adherent and floating cells were harvested and resuspended in 70% ethanol and stored at −20°C. Permeabilized cells were centrifuged and resuspended in PBS containing 20 mg/ml PI plus 40 mg/ml RNase (Sigma) for 30 min in the dark. Cells were then subjected to FACS analysis (FACScalibur, BD Bioscience, Bedford, MA) and gated for PI (*X* axis = FLH2; *Y* axis = events). Sub-G_1 cells were scored as apoptotic. Cells transfected with GFP-tagged constructs were fixed with 1% paraformaldehyde in PBS for 2 h at 4°C and permeabilized with 0.3% Triton in PBS for 20 min at room temperature. Permeabilized cells were then incubated with PI and RNase O/N at 4°C in the dark. Cells were gated for PI and GFP.

RESULTS

p73 Expression and Localization in Thyroid Cancer Cell Lines. To determine the expression of TAp73 in immortalized thyroid cancer cells, we analyzed 5 papillary, 3 follicular, and 7 anaplastic thyroid

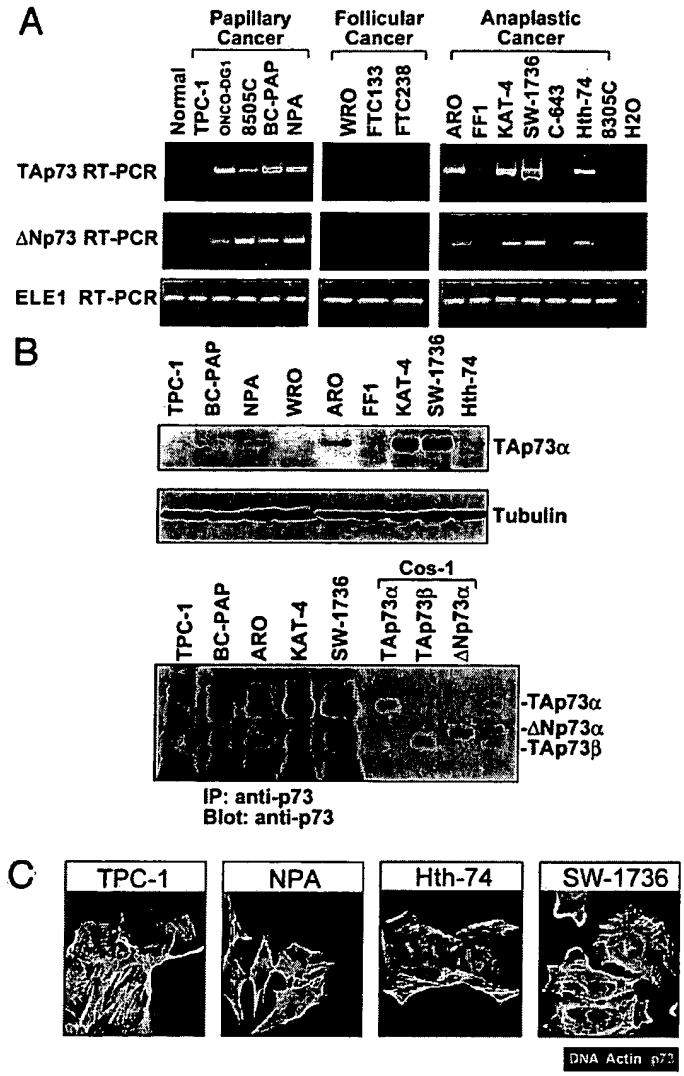

Fig. 1. p73 expression and localization in human thyroid cancer cell lines. *A*, thyrocites from a normal thyroid, 5 papillary (*TPC-1, ONCO-DG1, 8505C, BC-PAP,* and *NPA*), 3 follicular (*WRO, FTC133,* and *FTC238*), and 7 anaplastic (*ARO, FF1, KAT-4, SW-1736, C-643, Hth-74,* and *8305C*) thyroid cancer cell lines were screened by RT-PCR for TAp73 and ΔNp73 expression. Amplification of the ubiquitous *ELE1* gene confirms adequate normalization of the samples. *B*, lysates from representative thyroid cancer cells were subjected to SDS-PAGE and then probed with an antibody against NH_2-terminal residues present only in TAp73 (clone 429). The antitubulin blot shows equal protein loading in all conditions. To identify the ΔNp73 protein, cell lysates were immunoprecipitated with a mixture of three monoclonal antibodies recognizing all p73 isoforms. The lysates were then blotted with a polyclonal antibody (Ab5) against epitopes shared by both TA and ΔNp73. Cos-1 cells transfected with TAp73α, TAp73β, ΔNp73, or cotransfected with all three constructs are shown as a control. *C*, TPC-1, NPA, Hth-74, and SW-1736 cells were plated on coverslips, fixed, and stained for TAp73 (*red*) and filamentous actin (*green*). Nuclei were visualized with Hoechst (*blue*).

cancer lines by RT-PCR. We also screened 4 primary cultures of normal thyrocites. To detect all of the p73 isoforms derived from alternative splicing of the p73 NH_2 terminus (TA, Δ2, or Δ2–3 p73), we designed primers located on exon 1 and exon 5 of the p73 sequence. We did not find TAp73 transcripts in normal thyroid or in follicular thyroid cancer lines (Fig. 1*A*, *top panel*). However, 4 of 5 papillary and 4 of 7 anaplastic cell lines expressed variable levels of TAp73 (Fig. 1*A*, *top panel*). In contrast, we never observed amplicons compatible with Δ2p73 or Δ2–3p73 (data not shown). Because differential splicing of the p73 COOH terminus originates multiple TAp73 isoforms, we performed an additional RT-PCR, amplifying exons 10–14 of the *p73* gene. We found a high expression of p73α and lower but detectable levels of p73β (data not shown).

We then investigated whether our cell lines expressed ΔNp73. Using primers located on the last nucleotides of the third intron (transcribed only in ΔNp73) and on the fourth exon of the p73 sequence we observed the ΔNp73 mRNA in all of the cell lines positive for TAp73 (Fig. 1*A*, *bottom panel*).

We next sought to establish whether our RT-PCR results reflected p73 protein expression. We performed a Western blot on the thyroid cancer cells that expressed p73 mRNA, using an antibody (clone 429) that recognizes an epitope in the NH_2-terminal transactivation domain of p73. We confirmed protein expression in all of the cell lines positive for the TAp73 transcript (Fig. 1*B*, *top panel*) with the exception of 8505C cells (data not shown).

To ascertain ΔNp73 protein expression, thyroid cancer cells expressing ΔNp73 mRNA were lysed, immunoprecipitated, and blotted with antibodies that can distinguish all of the p73 variants by their molecular weight. We found barely detectable levels of ΔNp73 in BC-PAP and Hth-74 cells, whereas ONCO-DG-1, ARO, KAT-4, and SW-1736 expressed higher levels of ΔNp73 (Fig. 1*B*, *bottom panel;* data not shown).

We also evaluated the subcellular localization of TAp73 in thyroid cancer cells. An immunofluorescence experiment on three p73-positive cell lines (NPA, Hth-74, and SW-1736) using a TAp73-specific antibody (clone 429) revealed an intense nuclear signal (Fig. 1*C*). As expected, we did not observe p73 staining in the p73-negative TPC-1 cells (Fig. 1*C*).

Our initial findings indicated that TAp73 and ΔNp73 are not expressed in normal thyroid cells but are present in a wide variety of thyroid cancer cell lines, thereby raising the possibility that these proteins might be involved in thyroid tumorigenesis.

p73 Expression and Localization in Human Thyroid Cancer Specimens. To validate the results obtained in thyroid cancer cell lines, we analyzed the p73 status of tissue specimens derived from malignant tumors of the thyroid epithelium. Initially, we isolated mRNA from three thyroid cancers and a normal thyroid, and performed two RT-PCR reactions using primers specific for TAp73 or ΔNp73. TAp73 and ΔNp73 expression was elevated in all of the thyroid neoplasias but was undetectable in the normal thyroid tissue (Fig. 2*A*).

We then extracted cell lysates from 8 thyroid cancer specimens and performed an anti-p73 immunoblot. Six of 8 samples showed TAp73 protein expression, whereas 4 tumors expressed ΔNp73 (Fig. 2*B*).

To additionally confirm that the malignant thyroid cells were the source of p73 expression and to verify the subcellular distribution of TAp73 and ΔNp73, we analyzed a more consistent number of specimens by immunohistochemistry. We selected 20 thyroid specimens including 3 normal thyroids, 5 papillary, 4 follicular, and 8 anaplastic thyroid cancers (Table 1). TAp73 and ΔNp73 were never expressed in the normal thyroid (Fig. 2*C;* Table 1). On the contrary, we observed both TAp73 and ΔNp73 in all of the thyroid tumors tested (Fig. 2*C;* Table 1). This result was partially in contrast with our findings on follicular thyroid cancer cell lines in which we did not detect any p73 transcript. This discrepancy could be because of adaptation mechanisms proper of cell lines grown *in vitro*.

Immunohistochemistry also showed that TAp73 and ΔNp73 expression is predominantly nuclear, although in sporadic cases we could detect some citoplasmic staining.

Overall, our results in human thyroid tissue specimens confirmed that p73 is not expressed in normal thyroid epithelia but is present in thyroid cancer.

Lack of Functional Activity of TAp73 in Thyroid Cancer. p73 is a member of the p53 family of proteins and, like p53, can regulate cell proliferation and survival after DNA damage (32). Specifically, in response to DNA-damaging agents, TAp73 can induce several down-

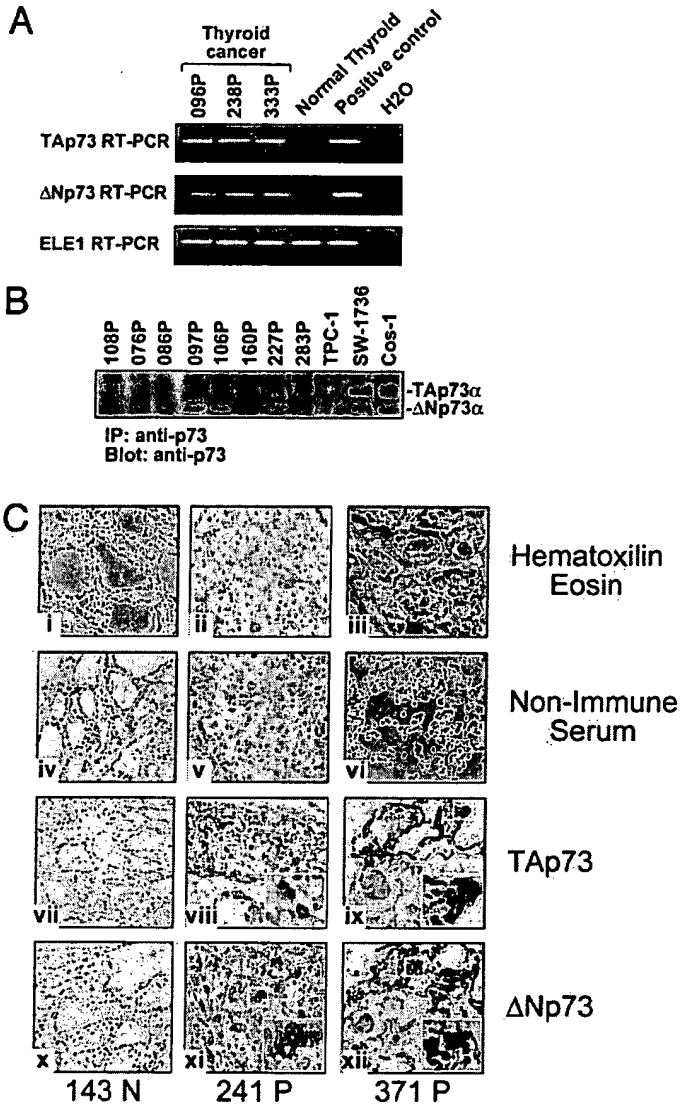

Fig. 2. TAp73 and ΔNp73 expression and localization in human thyroid cancer specimens *A*, specimens obtained from three thyroid cancers and a normal thyroid were analyzed by RT-PCR for TAp73 and ΔNp73 expression. RNA of SW-1736 cells was used as a positive control. The *ELE1* gene shows proper normalization of the RNA extracted from each sample. *B*, lysates from eight human thyroid cancer specimens were immunoprecipitated and then blotted with a polyclonal antibody (*Ab6*) that recognizes both TAp73 and ΔNp73. TPC-1 (p73-negative), SW-1736 (p73-positive), and COS1 cells transfected with both TAp73 and ΔNp73 were used as a control. *C*, immunohistochemistry for both TAp73 and ΔNp73 was performed on frozen thyroid cancer specimens (see Table 1). Hematoxilin Eosin staining (*i, ii,* and *iii*), a specificity control using a nonimmune serum (*iv, v,* and *vi*), TAp73 staining (*vii, viii,* and *ix*), and ΔNp73 staining (*x, xi,* and *xii*) of a normal thyroid tissue (*left*), and two thyroid carcinomas. Higher magnification of a selected area is shown in the insert.

stream targets including p21Cip1 and Bax. In turn, the increased expression of these proteins blocks the cell cycle in the G_1 phase or induces cell death. The observation that a protein with potential tumor-suppressor activity is highly expressed in thyroid tumors was difficult to understand and warranted additional investigation.

We have analyzed the p53 sequence of all of the thyroid cancer cells included in our study and report that each line presents mutations that functionally inactivate p53 (Table 2). Hence, in these cell lines, the induction of p21Cip1 or Bax in response to DNA damage is likely attributable to TAp73. We wanted to determine whether the tumor-suppressor activity of TAp73 was preserved in these thyroid cancer cells. Therefore, we selected seven representative lines and grew them for 12 h in normal medium or in medium additioned with 2 μM doxorubicin. Unexpectedly, doxorubicin treatment failed to cause any G_1 arrest in thyroid cancer cells (Fig. 3*A*). This result was additionally confirmed by a Western blot that failed to detect an increase in p21Cip1 expression in any of the thyroid cells exposed to doxorubicin (Fig. 3*C*, *top panel*). On the contrary, doxorubicin treatment increased p21Cip1 expression in HepG2 hepatocarcinoma cells that we chose as a control (Fig. 3*C*, *top panel*).

To assess whether p73 was able to induce apoptosis, we treated the seven cancer cell lines with 2 μM doxorubicin for 72 h. Whereas we noticed an increase in the total number of apoptotic cells, this increase was marginal at best (~10%), indicating that p73 is ineffective in eliciting apoptosis in thyroid cancer cells (Fig. 3*B*). This result was strengthened by an immunoblot with an anti-Bax antibody, showing minimal or no increase in the expression of this proapoptotic protein after doxorubicin treatment (Fig. 3*C*, *second panel from top*). However, doxorubicin treatment led to an induction of Bax in HepG2 control cells.

Table 1 *TAp73 and ΔNp73 expression in human thyroid cancer specimens*

Twenty specimens isolated from normal (3) or malignant (17) thyroid tissues were screened for TAp73 and ΔNp73 expression by immunohistochemistry. Two independent pathologists reviewed the slides separately. Quantification of the staining was based on the analysis of at least ten different fields.

	TAp73	ΔNp73
Normal Thyroid		
074N	−	−
081N	−	−
143N	−	−
Papillary Cancer		
321P	+++[a]	+++
340P	+++	+++
350P	++	++
360P	++	+++
380P	++	++
Follicular Cancer		
097P	++	++++
267P	+++	+++
283P	+++	+++
371P	+++	+++
Anaplastic Cancer		
006P	++++	++++
017P	+	++
096P	++	+++
103P	+++	+++
161P	+++	+++
238P	+++	+++
241P	++	++++
333P	+++	+++

[a] +, low expression; ++, moderate expression; +++, high expression; ++++, very high expression.

Table 2 *p53 mutations in thyroid cancer cell lines*

Total RNA isolated from the indicated thyroid cancer cell lines was subjected to RT-PCR with primers specific for p53. The expected amplicon was observed in all lines with the exception of SW-1736. Each PCR product was fully sequenced twice with an automated sequencer.

Cell line	p53 mutation
TPC-1[a]	K286E
ONCO-DG-1	R248Q
8505C[a]	R248G
BCPAP	D259Y; K286E
NPA	G266E; K286E(11)
WRO	P223L(41)
FTC-133	R273H(42)
FTC-238	R273H(43)
ARO[a]	R273H(11)
FF-1	E285K
KAT-4[a]	R273H(44)
SW-1736	null(44)
C643	R248Q; K286E
Hth-74	K286E
8305C	R273C

[a] Indicates cell lines that present the R to P polymorphism at codon 72.

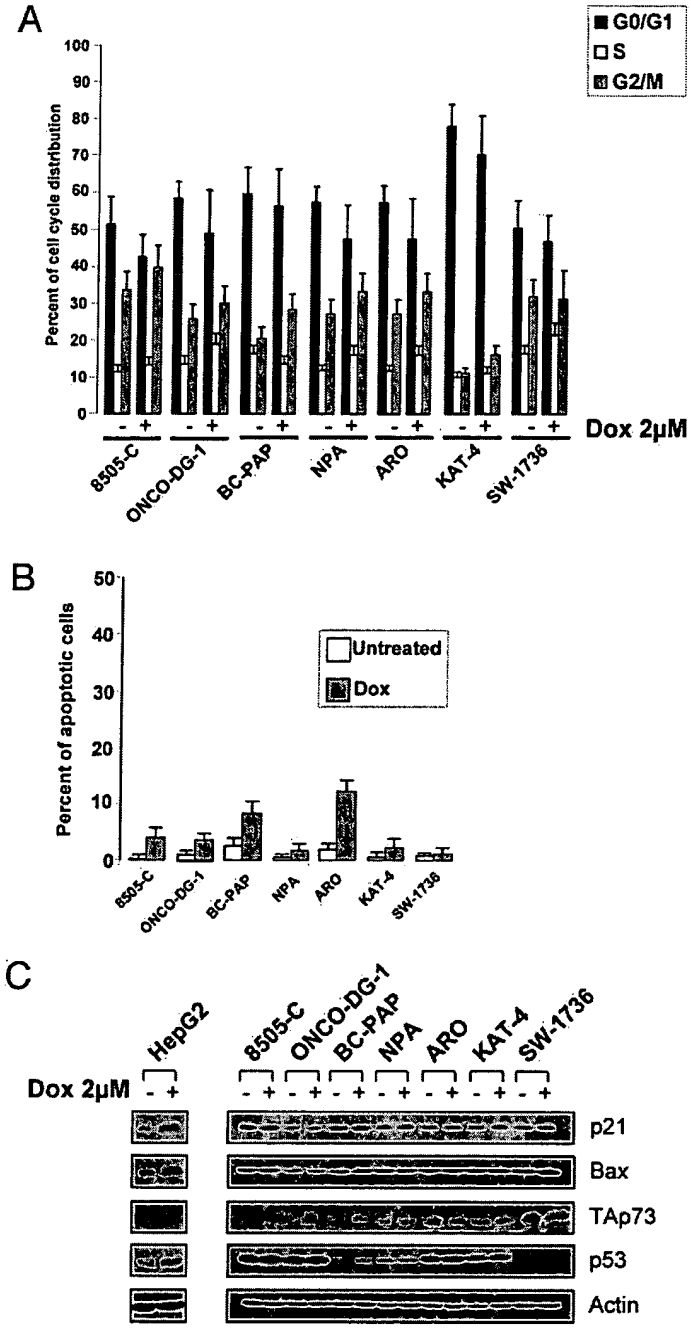

Fig. 3. The endogenous TAp73 lacks functional activity in thyroid cancer cell lines *A*, 8505-C, ONCO-DG-1, BC-PAP, NPA, ARO, KAT-4, and SW-1736 cells were analyzed for variations in their cell-cycle profiles, and in their viability before and after treatment with 2 μM doxorubicin. The *bars* represent average ± SD of the cell-cycle distribution after FACS analysis (G_0/G_1 ■, S □, G_2/M ▨) of four separate experiments. *B*, for the cell viability assay, untreated (□) or doxorubicin-treated (▨) cells were analyzed by FACS to evaluate the percentage of sub-G_1 cells in the total population. The *bars* represent average ± SD of three separate experiments. *C*, the above indicated thyroid cancer cells and the HepG2 hepatocarcinoma line (as a control) were grown for 12 h in normal medium or in the presence of 2 μM doxorubicin. At the end of treatment, the cells were lysed and analyzed by Western blot for the expression of p53, TAp73, and two downstream targets of the p53 family of proteins: p21Cip1 and Bax. The actin blot shows equal protein loading in all lanes (*bottom panel*).

In various epithelial cancer models, exposure to DNA-damaging agents leads to an induction of both TAp73 and p53. Indeed, doxorubicin treatment of HepG2 cells caused a strong induction of p53, whereas no TAp73 was detectable because these cells do not express p73 (Fig. 3*C*). We next performed an immunoblot with antibodies against TAp73 and p53 on lysates derived from thyroid cancer lines grown in the absence or the presence of doxorubicin. We did not observe significant modifications in TAp73 expression. Similarly, treatment with doxorubicin caused a mild increase of p53 levels in 8505C and BC-PAP cells but failed to increase p53 expression in the remaining thyroid cancer lines (Fig. 3*C, fourth panel from top*). It is likely that the failure of doxorubicin treatment to increase p53 levels in some of the thyroid cancer cells studied could be because of the high basal level of mutant p53 expressed by these cancer lines.

Our observations suggest that thyroid cancer cells do not induce TAp73 in response to doxorubicin treatment. This result could explain the inability of TAp73 to activate its target genes in thyroid neoplastic cells.

TAp73 Functional Activity Can Be Partially Restored by Overexpression. We wanted to determine whether overexpression of TAp73 could restore its ability to induce cell-cycle arrest or apoptosis. To track the cells overexpressing p73, we engineered a TAp73 construct that is tagged with both the HA epitope and the GFP. Both tags were inserted at the NH_2-terminal of TAp73α, upstream of the first codon, as described in the scheme depicted in Fig. 4*A*. To verify expression and localization of HA-GFP-TAp73, we transiently expressed this construct or a DNA encoding for wild-type TAp73 in Cos-1 cells. Immunoblots from lysates obtained from the transfected

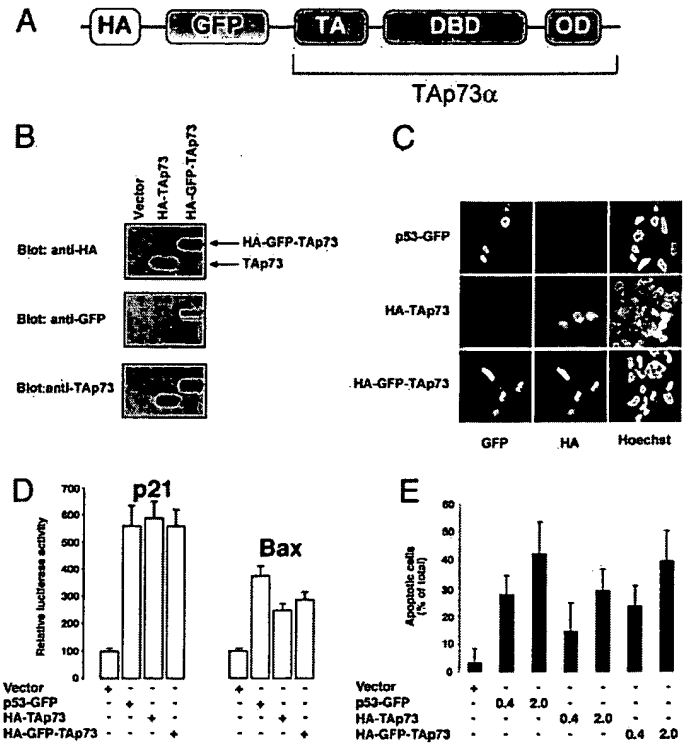

Fig. 4. Expression, localization, and biological activity of a TAp73 construct tagged with the GFP. *A*, to visualize cells transfected with TAp73, we engineered a TAp73 construct with a GFP tag. The GFP sequence was inserted in frame between the HA tag and the coding sequence for TAp73α as indicated in the diagram. *B*, HA-TAp73 or HA-GFP-TAp73 were ectopically expressed in Cos-1 cells. The cells were lysed and immunoblotted with anti-GFP or anti-p73 antibodies to verify the expression levels of the two different constructs. *C*, the Saos2 osteosarcoma cell line (p53 and p73 null) was transiently transfected with p53-GFP, HA-TAp73, or HA-GFP-TAp73. Two days after transfection, cells were fixed and stained with an anti-HA antibody (*red*). Nuclei were contrasted with Hoechst. *D*, Saos2 cells where cotransfected with reporter constructs for p21Cip1 or Bax and with the indicated p53 and p73 constructs. The next day, cells lysates were assayed for their relative luciferase activity expressed as fold activation with the control cells (*Vector*) arbitrarily set at 100. Results shown represent the average of three independent experiments normalized for transfection efficiency. *E*, Saos2 cells were transfected with an empty vector or with the indicated concentrations (μg of DNA) of p53-GFP, HA-TAp73, or HA-GFP-TAp73. After 24 h, cells were fixed and nuclei were stained with Hoechst. The percentage of cells with condensed chromatin was scored among the transfected cells.

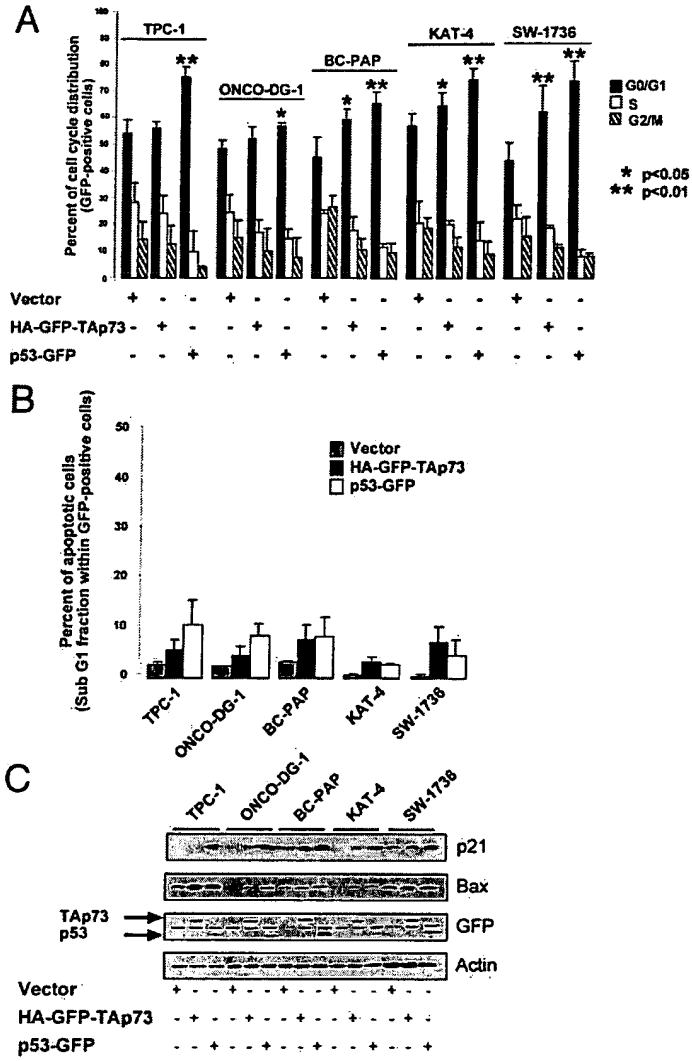

Fig. 5. p73 activity can be partially restored by overexpression *A*, TPC-1 (p73-negative), ONCO-DG-1, BC-PAP, KAT-4, and SW-1736 (p73-positive) cells were transfected with an empty vector, HA-GFP-TAp73 or p53-GFP. After transfection, the GFP-positive cells were analyzed by FACS to determine their cell-cycle profile. The bars (G_0/G_1 ■, S □, G_2/M ▨) represent average ± SD of three different experiments. Statistically significant variations are indicated with * (*, $P < 0.05$, **, $P < 0.01$). *B*, cell viability of the transfected population was determined as the sub-G_1 fraction identifiable by FACS analysis. The *bars* (empty vector: ▨, HA-GFP-TAp73: ■, p53-GFP: □) represent average ± SD of three separate experiments. *C*, TPC-1, ONCO-DG-1, BC-PAP, KAT-4, and SW-1736 were transiently transfected with HA-GFP-TAp73 or p53-GFP, and then analyzed by Western blot for the expression of p21Cip1, Bax, and GFP (to visualize either p73 or p53). An aspecific band can be seen in all conditions in the anti-GFP blot. The actin blot confirms equal protein loading in each lane.

cells confirmed that the two constructs could be expressed at similar levels (Fig. 4*B*). Immunofluorescence analysis also confirmed that HA-GFP-TAp73 localizes to the nucleus of Saos2 cells, an osteosarcoma cell line negative for both p53 and p73 expression (Fig. 4*C*). Furthermore, like p53-GFP and wild-type TA-p73, overexpression of HA-GFP-TAp73 in Saos2 cells activates the transcription of p21Cip1 and Bax (Fig. 4*D*). We finally tested the ability of our construct to induce apoptosis in the absence of p53. We transiently overexpressed increasing concentrations of p53-GFP, HA-TAp73, or HA-GFP-TAp73 in Saos2 cells and analyzed the transfected population by immunofluorescence to score cells with condensed chromatin and fragmented nuclei. The three constructs induced comparable levels of cell death (Fig. 4*E*).

We transfected HA-GFP-TAp73 in five thyroid cancer lines, one negative (TPC-1) and four positive (ONCO-DG-1, BC-PAP, KAT-4, and SW-1736) for p73 expression (Fig. 5*C*, *third panel from top*). The same cells were also transfected with an empty vector or with p53-GFP (Fig. 5*C*, *third panel from top*). FACS analysis showed that overexpression of HA-GFP-TAp73 in TPC-1 or ONCO-DG-1 cells had marginal effects in terms of cell-cycle distribution (Fig. 5*A*). However, in the three other cell lines, high levels of TAp73 induced a significant G_1 arrest (Fig. 5*A*). These findings were confirmed by an anti-p21Cip1 Western blot, showing an ample range of p21Cip1 induction in ONCO-DG-1, BC-PAP, KAT-4, and SW-1736 cells (Fig. 5*C*, *top panel*). In contrast, in TPC-1 cells p21Cip1 levels were not affected by TAp73 overexpression. In all of these cell lines, transient expression of p53-GFP was more potent than TAp73 in blocking the cell cycle in G_1 and inducing p21Cip1 (Fig. 5, *A* and *C*, *top panel*).

It remained to be verified whether high levels of HA-GFP-TAp73 could induce cell death in thyroid cancer cells. Thus, we analyzed the sub-G_1 population in the same thyroid cells transiently overexpressing HA-GFP-TAp73. Unlike what we observed for cell cycle progression, high levels of TAp73 produced no significant increase in apoptotic cells within the transfected population (Fig. 5*B*, *black bars*). The same was true for cells transfected with p53-GFP (Fig. 5*B*, *white bars*). Accordingly, when we performed an immunoblot on the transfected population, we noticed only a minimal increase in the expression levels of Bax (Fig. 5*C*, *second panel from top*).

Our findings indicate that TAp73 overexpression can restore the ability of the protein to arrest the cell cycle in G_1. However, as had been reported previously for p53 overexpression (33), high levels of TAp73 do not cause a significant increase in apoptosis.

Mutant p53 and ΔNp73 Contribute to TAp73 Inactivation in Thyroid Cancer. A possible explanation for the lack of TAp73 activity in thyroid cancer could be that TAp73 is inactivated frequently by mutational events. Whereas mutations of TAp73 are extremely infrequent in human neoplasias, such events have been described in tumors of the central nervous system, the lung, and the breast (16). Sequence analysis of the p73 mRNA isolated from all of the thyroid cancer cells studied failed to identify DNA alterations. Hence, we could exclude that mutations in the p73 sequence might account for its inability to arrest or kill thyroid cancer cells.

Previous reports have demonstrated that both mutant p53 and ΔNp73 play a role in the functional inactivation of TAp73 (27, 31). Inactivation of p53 is a frequent event in the progression of thyroid cancer to less-differentiated, more invasive phenotypes (11). Moreover, all of the thyroid cancer cell lines used in our study express mutated p53 (Table 2). To establish whether nonfunctional p53 is involved in TAp73 inactivation, we immunoprecipitated p53 in KAT-4 and Hth-74 cells, and then probed the blot with an anti-TAp73 antibody. Indeed, TAp73 was found to interact with mutant-p53 (Fig. 6*A*, *top panel*). As expected, we did not detect any interaction in TPC-1 and SW-1736 cells, because the former thyroid cancer line is negative for p73, whereas the latter does not express p53 (Fig. 6*A*, *top panel*).

To establish whether ΔNp73 is also involved in the functional inactivation of TAp73, we immunoprecipitated lysates from ARO, KAT-4, and SW-1736 cells with an antibody that recognizes only ΔNp73. Blotting with an antibody specific for TAp73 revealed that the endogenous ΔNp73 interacts with TAp73 (Fig. 6*B*). This interaction did not occur in TPC-1 cells that do not express p73 (Fig. 6*B*). These results were additionally confirmed by the opposite experiment in which an immunoprecipitation for TAp73 was followed by blotting with an antibody against ΔNp73 (data not shown).

Overall, our results confirmed that both mutant p53 and ΔNp73 are involved in the functional inactivation of TAp73 in thyroid cancer cells.

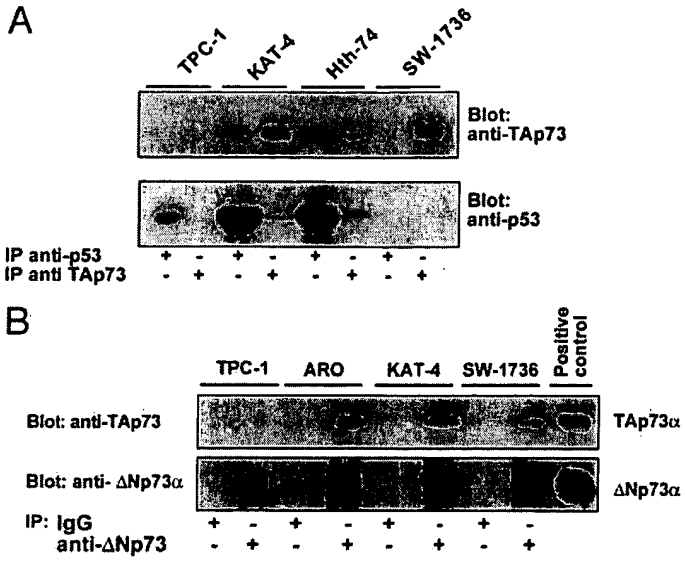

Fig. 6. Mutant p53 and ΔNp73 contribute to the inactivation of TAp73. *A*, lysates from TPC-1, KAT-4, Hth-74, and SW-1736 were immunoprecipitated with an anti-p53 antibody, and then blotted with an antibody specific for TAp73. Alternatively, cell lysates from the same cell lines were subjected to the reverse experiment, in which immunoprecipitation with an anti-TAp73 antibody was followed by a Western blot with an anti-p53 antibody. TPC-1 (p73-negative) and SW-1736 (p53-negative) cells were used as controls. *B*, lysates from TPC-1, ARO, KAT-4, and SW-1736 cells were immunoprecipitated with an antibody specific for ΔNp73 and then blotted using an antibody that recognizes only TAp73 (clone 429). Reblotting for ΔNp73 shows expression of this protein in the three positive cell lines (*bottom panel*). TPC-1 cells (p73-negative), and Cos-1 cells transiently expressing TAp73α and ΔNp73 are shown as a control.

Expression of Wild-Type p53 Down-Regulates Endogenous TAp73 in Thyroid Cancer. Previous evidence suggests that TAp73 is involved in the apoptotic response triggered by p53 (34). However, in thyroid cancer cell lines that express TAp73, high levels of wild-type p53 lead to G_1 arrest but not to cell death (Fig. 5*A*). We wanted to investigate whether, in our cell lines, overexpression of wild-type p53 influenced the levels of TAp73. Hence, we performed anti-TAp73 and anti-p53 immunoblots on two clones of ARO cells (AROIF and AROC5) that stably express a temperature-sensitive p53 construct. This naturally occurring p53 mutant is not functional at 37°C but is active at 32°C (33). Untransfected or mock-transfected ARO cells showed no variations in TAp73 expression at the two different temperatures (Fig. 7*A*, *top panel*). To our surprise, activation of p53 led to a significant decrease in TAp73 expression in both AROIF and AROC5 (Fig. 7*A*, *top panel*). As expected, p53 expression was not modified by growth at 32°C, because in these cells the lower temperature induces the functional activation but not the expression of the p53 protein (Fig. 7*A*, *second panel*). Activation of p53 also caused the induction of p21Cip1 (Fig. 7*A*, *third panel*). Notably, the up-regulation of p21Cip1 occurred in conditions in which TAp73 levels were down-regulated.

To investigate additionally the dynamics of the down-regulation of TAp73 in response to the expression of wild-type p53 we performed a time course experiment on ARO Neo and AROC5 cells grown at the permissive temperature for 1–4 days. A TAp73 immunoblot performed on the control ARO Neo cells showed no modifications in the expression of TAp73 (Fig. 7*B*). On the contrary, in AROC5 p73 levels began to decrease 24 h after the conformational activation of p53, and after 3 days p73 expression was virtually lost (Fig. 7*B*). Interestingly, the down-regulation of TAp73 was blocked when AROC5 cells were treated with the proteasome inhibitor MG132 (Fig. 7*B*). An anti-p21Cip1 immunoblot confirmed that p53 transcriptional activity was fully restored after 48 h of culture at 32°C (Fig. 7*B*, *right panel*). To gain additional insight on this unexpected result we transiently expressed p53-GFP in SW-1736 cells that present undetectable levels of endogenous p53. After transfection, the cells were analyzed by immunofluorescence for TAp73 expression. In most of the transfected cells we could not detect the TAp73 protein, indicating that expression of wild-type p53 led to a down-regulation of TAp73 (Fig. 7*C*). This observation was strengthened by the assessment of the percentage of p53-transfected cells that were also positive for TAp73. Only 21% of SW-1736 cells that expressed p53-GFP maintained visible expression of TAp73, as compared with 71% of the cells in which we overexpressed the unrelated H2B-GFP construct (Fig. 7*C*, *right panel*).

Our observations suggest that, in thyroid cancer cells, the expression levels of wild-type p53 and TAp73 are interdependent and possibly mutually regulated.

DISCUSSION

In this study we report that the p73 protein is not expressed in normal human thyrocites but is present in several histotypes of epithelial thyroid cancer. This observation is unexpected, because p73 is a member of the p53 family and exerts growth-inhibitory and apoptotic functions. Our results are even more puzzling considering that, in thyroid cancer cell lines exposed to DNA damage, TAp73 does not arrest the cell cycle nor does it induce cell death. These findings lead

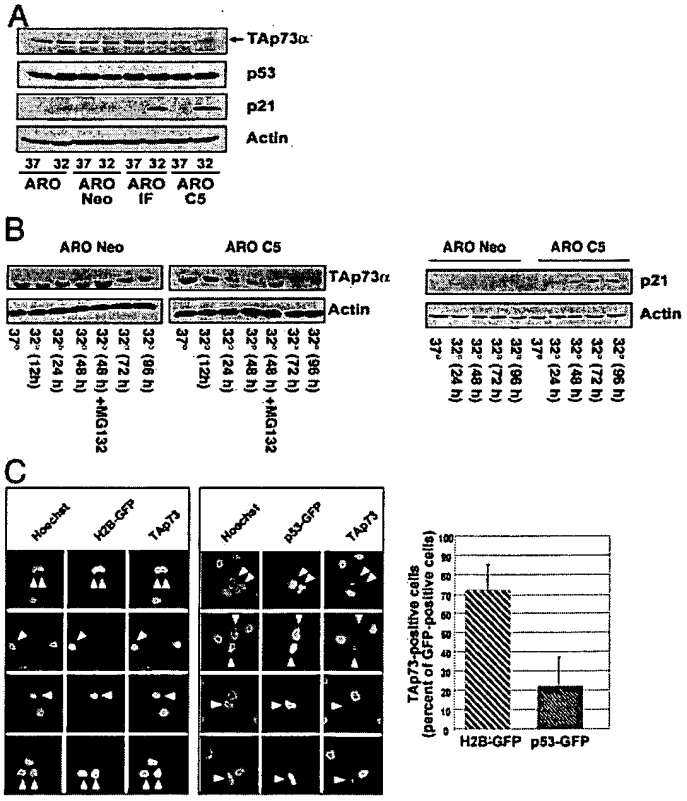

Fig. 7. Expression of wild-type p53 in thyroid cancer cell lines down-regulates TAp73 *A*, Two clones (*AROIF* and *AROC5*) of ARO cells stably transfected with a temperature-sensitive wild-type p53 construct (active at 32°C) were lysed and immunoblotted with antibodies against TAp73, p53, and p21Cip1. Untransfected (*ARO*) or mock-transfected (*ARO Neo*) cells were used as a control. The anti-actin immunoblot confirms equal protein loading in all of the lanes. *B*, ARO Neo (*control*) and AROC5 cells grown at 37°C or at 32°C were lysed at the indicated time points and immunoblotted with an anti-p73 or an anti-p21Cip1 antibody. Where indicated, the proteasome inhibitor MG132 was added to the cells 12 h before they were collected. An actin reblotting shows equal protein loading in each condition. *C*, SW-1736 cells were transiently transfected with H2B-GFP or p53-GFP and then stained with an antibody against TAp73 (*red*) and with Hoechst to visualize the nuclei (*blue*). The *bar graph* represents the quantification of the TAp73-positive cells in the transfected population (300 cells were counted for each condition).

to ask why the TAp73 protein of thyroid cancer cells fails to exercise its tumor-suppressor functions.

Many different mechanisms could explain our observations. First of all, it is interesting to note that in thyroid cancer cells, TAp73 is unresponsive to doxorubicin, because exposure to the drug leads to a marginal increase in protein expression. We are still uncertain if this lack of TAp73 induction is because of a dysfunctional promoter or to a decreased half-life of the protein. In any case, this phenomenon is not specific for doxorubicin because many other DNA-damaging agents fail to induce TAp73 expression in our panel of thyroid cancer lines.[5] Moreover, the fact that overexpression can only partially rescue TAp73 tumor-suppressor activity suggests that, in addition to defects upstream of TAp73 activation, signaling downstream of TAp73 could be compromised as well. Hence, thyroid cancer cells appear to select multiple roadblocks to interfere both with the activation and the downstream signaling of TAp73.

We report elsewhere that one such block is represented by a strong reduction in the nuclear import of the ABL tyrosine kinase (35). Here we describe two additional mechanisms involved in the inactivation of TAp73 in thyroid cancer cells. The first concerns the p53 tumor suppressor protein. Previous studies have demonstrated that mutant p53 can interact with TAp73 via its core domain (36). The formation of heterotetramers composed of mutated p53 and wild-type TAp73 results in the functional inactivation of the latter (30, 31). All of the thyroid cancer cells included in our study express a nonfunctional p53 protein (Table 2). In several of them we have found mutant p53 to interact with TAp73.

A second mechanism that interferes with TAp73 activity in thyroid cancer involves ΔNp73. ΔNp73 is a dominant-negative form of p73 that can abrogate the activity of TAp73 (16). In our study we have found that thyroid cancer cell lines that express TAp73 also express ΔNp73. Immunoprecipitation studies documented a direct interaction between TAp73 and ΔNp73. These results strongly suggest that ΔNp73 may also play a major role in the functional inactivation of TAp73.

An issue that is still unresolved concerns the biological role of p73 in the malignant thyroid epithelium. In our study we identified p73 in a wide panel of thyroid cancer cell lines and in all of the primary specimens isolated from thyroid cancer. On the contrary, the normal thyroid did not express p73. Thus the question lingers: why do thyroid cancer cells express p73? Whereas it is true that all tumor cells are able to survive by dodging the many death signals that surround them (37), it seems unlikely that in thyroid cancer p73 expression is a remnant of an apoptotic pathway that the cells have successfully inactivated. Previous reports suggest that, in ovarian cancer, p73 expression reduces p53 transcriptional activity (38, 39). More recently, Freebern *et al.* (40) have reported that in a leukemia cell line, TAp73, acts as a transcriptional repressor, blocking p53-mediated cell death. Therefore, it is possible that p73 expression could confer some kind of selective advantage to thyroid cancer cells. Indeed, in our cancer lines, overexpression of wild-type p53 causes a strong down-regulation of TAp73 expression. This down-regulation is due to an increased degradation of the protein, because treatment with a proteasome inhibitor can restore normal levels of TAp73. It remains to be seen whether the down-regulation of TAp73 after ectopic expression of wild-type p53 is because of p53 itself, or represents an adaptation response of the neoplastic cells to avoid a synergic effect between p53 and TAp73.

An additional level of complexity is represented by the expression of ΔNp73 regardless of the histotype or the differentiation status of thyroid neoplasias. Wild-type p53 and TAp73 have both been identified as targets of ΔNp73 biological activity. Differentiated thyroid carcinomas usually display wild-type p53. Hence, it is possible that in these tumors ΔNp73 disrupts p53 activity. On the other hand, undifferentiated thyroid cancers are often characterized by the inactivation of both p53 alleles. It remains to be determined whether, in this aggressive form of thyroid carcinoma, TAp73 is the only target of ΔNp73.

In summary, whereas it seems clear that p73 expression is a marker of neoplastic thyroid cells, the biological function and the molecular interplay of the various p73 isoforms in thyroid cancer remain partially unresolved. The clarification of these complex issues awaits additional investigation.

[5] Frasca and Vigneri, unpublished observations.

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p73 Expression in Human Normal and Tumor Tissues: Loss of p73α Expression Is Associated with Tumor Progression in Bladder Cancer

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ABSTRACT

Purpose: **To characterize the expression profile of p73 in human normal tissues by immunohistochemistry (IHC) and to analyze the correlation between p73 expression and bladder cancer progression.**

Experimental Design: **CJDp73 was characterized for p73α detection in Western blot and IHC through its application to isoform-transfected 293 cells. Normal tissues were analyzed by IHC with the CJDp73 antiserum. Transitional cell carcinoma (TCC)-derived cell lines were subjected to reverse transcription-PCR and Western blot. TCC tissue microarrays were analyzed for p73α expression by IHC, and the results were statistically analyzed.**

Results: **p73 immunostaining was nuclear and restricted to epithelial cells of certain organs such as squamous epithelium of the epidermis and transitional epithelium of the bladder. The expression was also observed in certain specialized glandular epithelia such as acinar cells of breast and parotid gland. Four of seven TCC-derived cell lines had low to undetectable p73α protein levels. We found undetectable or low p73α expression in 104 of 154 (68%) TCC cases, this phenotype being more frequently observed in invasive tumors when compared with superficial lesions. This association was statistically significant ($P < 0.0001$). We also observed a significant association between p53, p63, and p73α alterations with bladder cancer progression ($P < 0.0001$).**

Conclusions: **p73α plays a tumor suppressor role in bladder cancer, and its inactivation occurs through an epigenetic mechanism, most probably involving protein degradation.**

INTRODUCTION

p73 was identified as the first homologue of p53 and along with p63 have added an additional level of complexity to the analysis of p53 function (1, 2). The *p73* gene maps to 1p36.33, a region reported to harbor frequent deletions in certain human tumors, including neuroblastoma and colon cancer (3, 4). Three different promoters (P1, P2, and P3) have been described for *p73* at the RNA level, giving rise to either the TA (transcriptionally active) or ΔN (dominant-negative) variants (5). Additionally, *p73* is subject to extensive COOH-terminal alternative splicing of exons 11 through 14, yielding at least six isoforms (6–8).

p73 shares significant amino acid identity to p53 in the transactivation, DNA binding, and oligomerization domains (6). Not surprisingly, TAp73 can transactivate many p53 target genes such as *p21* and *BAX*, leading to cell cycle arrest or apoptosis, respectively (6–11). In opposition, ΔNp73 acts as dominant-negative inhibitor, not only toward TAp73, but also toward other members of the p53 family (2).

Initially, because of its similarities to *TP53* and the chromosomal location in a region frequently deleted, *p73* was hypothesized to be a suppressor gene. This observation has been challenged by the low frequency of *p73* mutations found in primary tumors, suggesting that the tumor suppression activity of *p73* could be lost epigenetically and is not conforming to Knudson's two-hit hypothesis (4). A recent study demonstrated that the simultaneous absence of p73 and p63 affected the induction of p53-dependent apoptosis in response to DNA damage in E1A-expressing cells and in the developing mouse central nervous system (12). However, p73-deficient mice were not tumor prone or display an increase in tumor incidence (13).

Previous studies have shown that *p73α* and *p73β* transcripts are ubiquitously expressed at very low levels in some normal human organs, including brain, heart, kidney, liver, and spleen (14). Bladder cancer is the fifth most common cancer in the United States (15). Alterations in proto-oncogenes such as *Ras* (16) and alterations of certain tumor suppressors such as *TP53* and *RB* (17) have been associated with bladder cancer progression. Approximately 50% of bladder tumors harbor *TP53* mutations or an altered p53 expression (18). A previous study from our group, centered in a closely related set of patients to the here described, found p53 nuclear overexpression and *TP53* mutations in ~50% of the cases. p53 overexpression, associated with loss of p21 and mdm2 nuclear overexpression, was found to be a significant prognostic factor associated with patient survival (19). More recently, we reported the significant

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Table 1 p73α expression in normal adult tissues as determined by immunohistochemistry

Organ site	Score[a]	Positive component
Digestive tract		
Esophagus	(++)	Basal and parabasal layers of squamous epithelium
Stomach	(−)	
Small intestine	(−)	
Colon	(±)	Epithelial cells in the colonic crypts
Glands associated with digestive tract		
Liver	(−)	
Pancreas	(+)	Exocrine pancreatic acini
Gall Bladder	(−)	
Parotid[b]	(+)	Basal ductal cells
Endocrine system		
Adrenal	(±)	Zona granularis
Thyroid	(−)	
Lymphoid system		
Lymph node	(−)	
Spleen	(−)	
Tonsil[b]	(++)	Basal and parabasal layers of squamous epithelium
Thymus	(−)	
Locomotor system		
Skeletal striated muscle	(−)	
Bone[b]	(−)	
Ligament[b]	(−)	
Reproductive system (female)		
Breast	(+)	Basal ductal and acinar cells
Endocervix	(−)	
Exocervix	(+)	Basal layer of squamous epithelium
Endometrium	(−)	
Ovary	(−)	
Placenta	(−)	
Reproductive system (male)		
Prostate	(+)	Basal ductal and acinar cells
Testis	(++)	Spermatogonia
Respiratory system		
Larynx[b]	(++)	Basal cells
Lung	(−)	
Skin		
Epidermis	(+)	Basal and parabasal layers of squamous epithelium
Hair follicles	(+)	Basal and parabasal layers of squamous epithelium
Sweat glands	(+)	Basal cells
Urinary system		
Bladder	(++)	Transitional epithelium (including umbrella cells)
Kidney	(−)	

[a] −, undetectable, ±; occasional, +; moderate, ++; strong.
[b] IHC using OCT-embedded frozen tissue.

association between loss of p63 expression and tumor progression in bladder cancer, analyzing the same cohort of patients studied here. p63 expression is lost in most invasive bladder TCC,[3] whereas papillary superficial tumors maintain p63 expression (20).

In this study, we analyzed the expression of p73 in human normal tissues at the protein level. We also examined p73α expression patterns in a cohort of patients with bladder tumors previously examined for p53 and p63 and studied the relationship between bladder cancer progression and the loss of p73α expression, both individually and related to p63 expression and p53 status. A role for p73 as a tumor suppressor is hypothesized for bladder cancer.

PATIENTS AND METHODS

Human Tissue and Patient Characteristics. A broad spectrum of paraffin-embedded normal human tissues were studied for the expression of p73 at the protein level (Table 1). Normal tissues used for RNA extraction were embedded in cryopreservative solution OCT (Miles Laboratories, Elkhart, IN), snap frozen, and stored at −80°C. Representative H&E-stained sections of each frozen and paraffin blocks were examined microscopically to confirm the presence of normal tissue in the samples. Tumors from 154 patients with TCC of the bladder were also analyzed. Tumor specimens included 49 papillary superficial tumors (T_a and T_1) and 105 invasive lesions (T_2–T_4). These cases were coded to ensure patient confidentiality.

[3] The abbreviations used are: TCC, transitional cell carcinoma; RT-PCR, reverse transcription-PCR; IHC, immunohistochemistry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

CJDp73 Antibody Production. A 518-bp DNA fragment, spanning amino acids 428–599 and mapping to the COOH terminus of simian p73α, was inserted downstream in frame of the polyhistidine (6xHIS) tag of the pRSET-C-T7 expression vector (Invitrogen Life Technologies, Inc., Carlsbad, CA). The p73α fusion protein was expressed in BL21(DE3) *Escherichia coli* and purified on an 8% SDS-polyacrylamide gel. Crushed gel slices containing the fusion protein were used to raise polyclonal antibodies in rabbits (Covance Research Products, Inc., Denver, PA). The resultant hyperimmune serum was designated CJDp73 and an aliquot was affinity purified using recombinant 6xHIS-p73α protein attached to a Nickel-NTA column (21).

Cell Lines. Nine bladder cancer cell lines were studied, including RT4, which is derived from a superficial TCC; T24, J82, 5637, HT-1197, HT-1376, UM-UC-3, and TCC-SUP derived from invasive TCC; and SCaBER derived from an invasive squamous cell carcinoma of the bladder. For transfection experiments, transformed human primary embryonal kidney 293 cells were used. Bladder tumor-derived cell lines and 293 cells were obtained from and maintained as recommended from the American Type Culture Collection (Manassas, VA). H24 cell lines expressing tetracycline-repressible simian HA-p73α and murine myc-ΔNp63α (for use in Western blots and RT-PCR) were a gift from Xinbin. Chen (22). These stable cell lines do not express p63 or p73 when grown in the presence of 1 μg/ml tetracycline.

Plasmids, Cell Culture, and Transfection. For CJDp73 characterization by Western blotting, transformed human primary embryonal kidney 293 cells were transfected in 35-mm dishes with human HA-tagged *p53*, *TAp73α*, *TAp73β*, *TAp73γ*, *TAp73δ*, *ΔNp73α*, *ΔNp73β*, *ΔNp73γ*, and vector control (pcDNA3) by the Fugene method (Roche Diagnostics, Indianapolis, IN). After 48 h, the cells were washed with a PBS solution and pelleted by centrifugation. For the CJDp73 characterization by IHC, transformed human primary embryonal kidney 293 cells were transfected in 2-well chambered slides (Nalge Nunc International, Naperville, IL) with *TAp73α*, *TAp73β*, *TAp73γ*, *TAp73δ*, *ΔNp73α*, *ΔNp73β*, *ΔNp73γ*, and vector control (pcDNA3). After 48 h, the slides were washed twice with PBS, fixed for 10 min in formalin, washed again with PBS, and subjected to the IHC protocol described below.

Tissue Array Construction. Normal and tumor tissues were fixed with formalin and embedded in paraffin. Five-μm sections stained with H&E were obtained to identify viable, representative areas of the specimen. From the defined areas core biopsies were taken with a precision instrument (Beecher Instruments, Silver Spring, MD), as described previously (23). Tissue cores with a diameter of 0.6 mm from each specimen were punched and arrayed in triplicate on a recipient paraffin block (24). Five-μm sections of these tissue array blocks were cut and placed on charged poly-lysine-coated slides. These sections were used for immunohistochemical analysis (25). Cell lines known to express p73 were used as positive controls (see above).

IHC. Five-μm tissue sections were deparaffinized, rehydrated in graded alcohols, and processed using the avidin-biotin immunoperoxidase method. Briefly, sections were submitted to antigen retrieval by microwave oven treatment for 15 min in 10 mM citrate buffer (pH 6.0). Slides were subsequently incubated in 10% normal serum for 30 min followed by the overnight incubation at 4°C with the appropriately diluted primary antibody. The rabbit antisimian CJDp73 polyclonal antibody (~50 ng/μl) was used at a 1:400 dilution to a final concentration of 0.125 ng/μl. The mouse monoclonal antihuman p53 antibody (PAb1801 clone) recognizing an epitope located between amino acids 46 and 55 was used at 0.2 μg/ml (Oncogene Research Products, Boston, MA). p63 was analyzed with the monoclonal 4A4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (20). After the primary antibody, samples were incubated with the biotinylated antirabbit or antimouse immunoglobulins for 30 min, followed by avidin-biotin peroxidase complexes for 30 min (Vector Laboratories, Inc., Burlingame, CA). 3,3′-Diaminobenzidine was used as the chromogen and hematoxylin as the nuclear counterstain. Slides were reviewed by several investigators (P. P., P. C., M. D., C. C-C., and C. J. D.). Some normal tissues were only available as frozen samples. These sections were cut, fixed on formalin for 10 min at 4°C, and analyzed as the paraffin embedded samples, except for the deparaffinization step.

Results were scored in TCC lesions by estimating the percentage of tumor cells showing characteristic nuclear staining. An arbitrarily defined 10% cutoff was taken to classify the TCC data into two categorical groups (positive *versus* negative). TCC samples were considered to have a negative phenotype for the p73α expression when $<10\%$ of nuclei showed staining. The cutoff point for p53, p53 $\geq$ 20% immunoreactive tumor cells, was selected based on previous publications (19). The cutoff point for p63 was chosen at 30% of tumor cells displaying nuclear immunostaining (20).

Western Blotting. For Western blotting, total cell extracts of cultured cells were prepared as described previously (26). The rabbit antisimian CJDp73 polyclonal antibody was used at 1:500 dilution. The anti-HA monoclonal antibody (HA.11) was obtained from Covance (Princeton, NJ) and used at 1:1000 dilution. The anti-myc monoclonal antibody (S1826) was obtained from Clontech (Palo Alto, CA) and used at 1:500 dilution. The goat anti-human Ran polyclonal antibody (C-20, sc-1156) was purchased from Santa Cruz Biotechnology and used at a dilution of 1:1000. Horseradish peroxidase-conjugated antibodies (Amersham, Arlington Heights, IL, and Sigma, St. Louis, MO) were used as secondary antibodies at a 1:1000 dilution. Proteins were visualized with an enhanced chemiluminescence plus detection system (Amersham).

RNA Isolation and RT-PCR. Total RNA was extracted from the nine bladder carcinoma cell lines using TRIzol (Invitrogen Life Technologies, Inc.) according to manufacturer's instructions. Total RNA (250 ng) was then amplified using *p73* isoform-specific primers using the Superscript One-Step RT-PCR kit with Platinum Taq (Invitrogen Life Technologies, Inc.) using the manufacturer's protocol (50 μl of reaction volume). All reverse transcription reactions were carried out for 30 min at 50°C, then 4 min at 94°C, followed by isoform-specific PCR conditions for each primer set: A, *TAp73* isoforms (nucleotides 61–366 of *TAp73α*): 40 cycles at 94°C (30 s), 56°C (40 s), and 72°C (30 s) using SKO53 (sense, 5′-TCTCTGGAACCAGACAGCAC-3′) and SKO54 (antisense, 5′-GGGGTAGTCGGTGTTGGAG-3′); B, *ΔNp73* isoforms (nucleotides 5–218 of

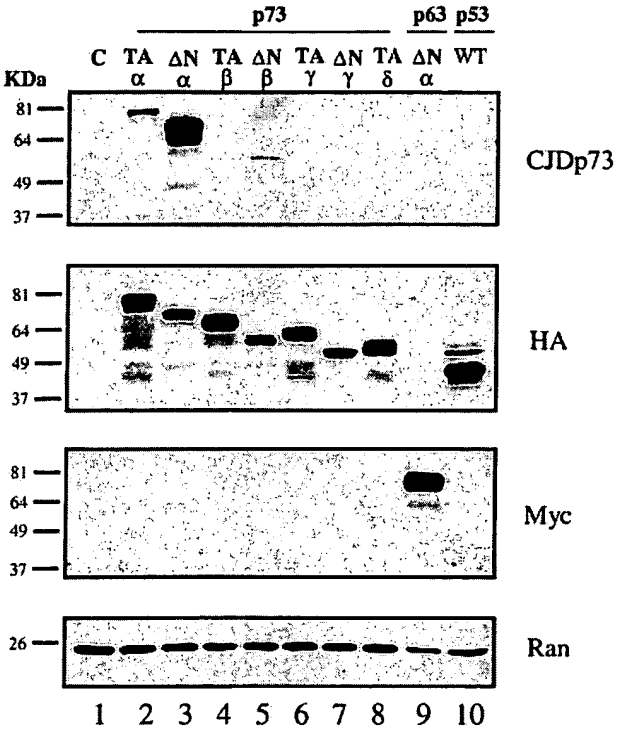

Fig. 1 p73 expression levels in 293-transfected cells. A panel of antibodies, including CJDp73, was used in Western blots of protein extracts from transfected 293 cells. In the first lane, "C" identifies transfected cells with the vector control HA-tagged. The next seven lanes depict the expression of seven different HA-tagged isoforms of p73 following this order from *Lane 2* to *Lane 8* (TAp73α, ΔNp73α, TAp73β, ΔNp73β, TAp73γ, ΔNp73γ, and TAp73δ). Two controls were added to ensure the specificity of the detection; *Lanes 9* and *10* (myc-tagged ΔNp63α and HA-tagged p53). The *bottom panel* shows the anti-Ran antibody used in blots from protein extracts at an equivalent loading through all lanes. The *middle panels* illustrate the set of p53 family members transiently expressed on 293 cells. ΔNp63α is the only myc-tagged protein showed after anti-myc blotting, whereas the other p53 family members are depicted when using the anti-HA antibody. Please note the specificity of CJDp73 antibody for the two p73α isoforms and ΔNp73β, not reacting with ΔNp63α or p53.

ΔNp73): 40 cycles at 94°C (30 s), 60°C (20 s), and 72°C (8 s) using SKO78 (sense, 5′-TGTACGTCGGTGACCCCG-3′) and SKO54 (antisense, 5′-GGGGTAGTCGGTGTTGGAG-3′); C, *p73α* COOH-terminal variant (nucleotides 1591–1897 of *TAp73α*): 40 cycles at 94°C (30 s), 56°C (40 s), and 72°C (30 s) using SKO57 (sense, 5′-CTGAAGATCCCCGAGCAGTA-3′) and SKO58 (antisense, 5′-CTCCGTGAACTCCTCCTTGA-3′); D, *p73β* and *p73∂* COOH-terminal variants (nucleotides 899-1203 of *TAp73∂*): 40 cycles at 94°C (30 s), 56°C (40 s), and 72°C (30 s) using SKO61 (sense, 5′-GACCGAAAAGCTGAT-GAGGA-3′) and SKO62 (antisense, 5′-CCCCAGGTCCTCTG-TAGGAG-3′); and E, *p73γ* COOH-terminal variant (nucleotides 1198–1411 of *TAp73γ*): 40 cycles at 94°C (30 s), 54°C (4 s), and 72°C (6 s) using SKO59 (sense, 5′-CGGGATGCTCAA-CAACCAT-3′) and SKO60 (antisense, 5′-TGCAGGTGGTA-AATGCTCTG-3′). The *GAPDH* gene was chosen as an endogenous expression RT-PCR standard using SKO36 (sense, 5′-GAAGGTGAAGGTCGGAGT-3′) and SKO37 (antisense, 5′-GAAGATGGTGATGGGATTTC-3′). Isoform-specific RT-PCR (including *GAPDH* and a no-RNA control) was performed in triplicate. Forty-five μl of RT-PCR products were resolved in 2.0% agarose gels. Total RNA from H24 cells expressing HA-TAp73α was used as positive control.

Statistical Analyses. In this study, the association between p73α expression levels and histopathological variables of the cases analyzed was evaluated. p73α expression was treated as both a continuous variable and a categorical variable (positive *versus* negative taking 10% of positive cells as the cutoff point). p63 (positive *versus* negative) and p53 (wild-type *versus* mutant) were treated as categorical variables. These determinations were made as a final call at the tumor, as opposed to the core, level. The difference on tumor stages between groups split according to data from p53, p63, and p73α staining was analyzed as categorical variables. p73α expression as a categorical variable was also individually analyzed *versus* tumor stage and grade. A Fisher's exact test was used to test the hypothesis of no difference between the categorical groups analyzed. When treating the p73α expression as continuous variable, each tumor was treated as a random effect to account for the inherent but unknown correlation between the different cores of an individual tumor. The data were then fit using the method of restricted maximum likelihood, and an F test was used to test the hypothesis of no difference between groups.

RESULTS

Characterization of the CJDp73 Antibody. A p73α fusion protein was used for immunization of rabbits by standard methods and an aliquot of the resultant hyperimmune serum, designated CJDp73, was affinity purified (see "Materials and Methods"). Western blot analysis of 293 cell extracts ectopically

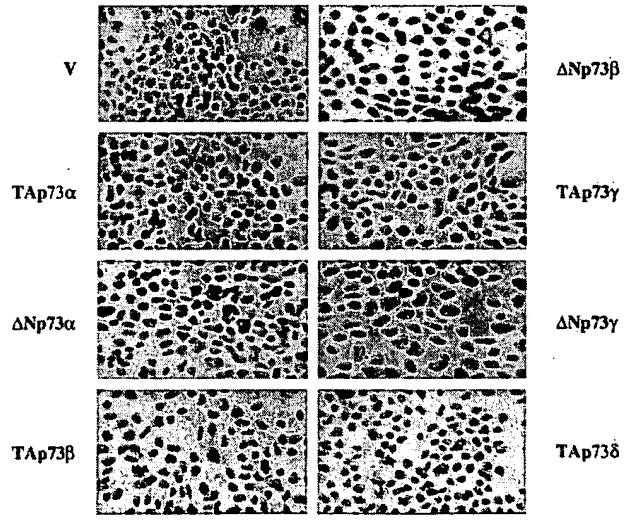

Fig. 2 Immunohistochemical analyses of p73 expression using CJDp73 antibody on transiently transfected 293 cells. Transient transfections of 293 cells were performed using seven HA-tagged p73 isoforms (TAp73α, ΔNp73α, TAp73β, ΔNp73β, TAp73γ, ΔNp73γ, and TAp73δ) and the control vector ("V"). p73α, TA, and ΔN isoforms are the major products detected by the CJDp73 antibody, as revealed by their intense nuclear immunoreactivity.

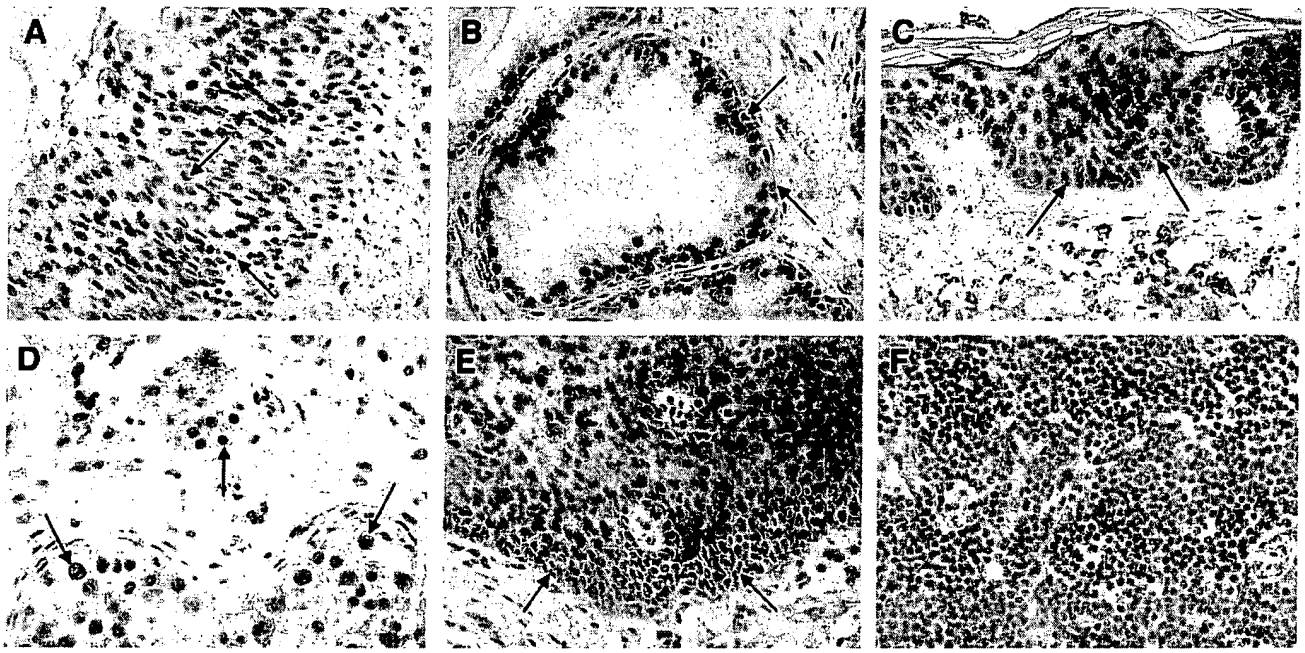

Fig. 3 Immunohistochemical analyses of p73α expression using CJDp73 antibody in normal human tissues. *A*, normal bladder urothelium showing nuclear reactivities for p73α throughout the transitional epithelium. *B*, p73α nuclear immunostaining was detected in the basal cells surrounding prostatic ducts and acini. *C*, intense nuclear immunoreactivities were found in the keratinocytes lining the basal cell layer of the skin epidermis. *D*, section of testis illustrating spermatogonia cells displaying intense nuclear staining. *E*, the basal cell layer of the esophageal mucosa also showed intense nuclear immunoreactivities. *F*, p73α immunoreactivities were not detected on lymphocytes such as those from this section of a lymph node. Original magnifications: *A–E*, ×200; *F*, ×100.

expressing HA-p73, HA-p53, myc-p63, and vector control constructs demonstrated that CJDp73 recognized both TAp73α and ΔNp73α, as well as ΔNp73β but to a lesser extent (Fig. 1). Interestingly, although CJDp73 reacted weakly with ΔNp73β, this antibody does not react with TAp73β (Fig. 1). As expected, CJDp73 does not react with p73γ and p73δ (Fig. 1). More importantly, CJDp73 does not recognize p53 and ΔNp63α (Fig. 1).

Immunoreactivity of CJDp73 was also examined in tissues and cell lines by IHC. We first titrated the CJDp73 antibody on human normal skin sections and obtained an optimal concentration for IHC of 0.125 ng/μl. At this concentration, the skin displayed intense p73 nuclear immunoreactivity in cells of the basal and parabasal layers of the epidermis, hair follicles, and sweat glands (Table 1). We next examined 293 cells ectopically expressing the seven p73 constructs used in the above Western analysis. Only those cells expressing TAp73α and ΔNp73α displayed intense p73 nuclear immunoreactivity with CJDp73 (Fig. 2). The other p73 isoforms and vector control showed a lack of immunoreactivity (Fig. 2). CJDp73 was raised against p73α and p73β isoforms, the antiserum being mainly directed to the α-derived isoforms, as revealed by both Western blot and IHC. The affinity for the β isoforms is much lower, and it appears to be only over the detection limit of immunoblotting for the ΔNp73β isoform. Transient transfection experiments for IHC and Western blot were conducted in triplicate and in parallel. In all cases, the ΔNp73β isoform was detected by Western blot but not by IHC. Thus, we conclude that CJDp73 is an appropriate reagent to specifically discriminate between p73α and other p73 isoforms by IHC assays.

Expression of p73α in Stratified, Transitional, and Simple Epithelia. In contrast to wild-type p53, where protein expression is rarely detectable by immunohistochemical methods but is present at low levels in all cell types (27), we found a relatively restricted tissue-specific distribution of TA- and ΔN-p73α in normal tissues using the anti-p73 polyclonal antibody CJDp73. Table 1 summarizes the patterns of p73α expression in normal tissues.

We observed that the stratified squamous epithelia studied, which included the epidermis of the skin and tonsil mucosa, displayed intense p73α nuclear immunoreactivity in cells of the basal layer, moderate to weak immunoreactivity in the parabasal layers, and undetectable staining in the more terminally differentiated and superficial cell layers (Fig. 3). Stratified nonsquamous epithelium such as that of the esophageal mucosa showed a similar pattern of staining, with intense immunoreactivity found in basal cells. Columnar epithelium of larynx and upper bronchi showed strong staining in basal cells. The transitional epithelium, lining the calyces of the kidney, ureters, bladder, and parts of urethra, showed intense immunoreactivity of all layers, including umbrella cells (Fig. 3). Certain glandular epithelial cells in specific organs such as the acini and ducts of the breast and prostate displayed moderate to intense p73α nuclear staining (Fig. 3). Additionally, we observed moderate staining in the parotid gland and occasional staining in cells of the crypts of the colon.

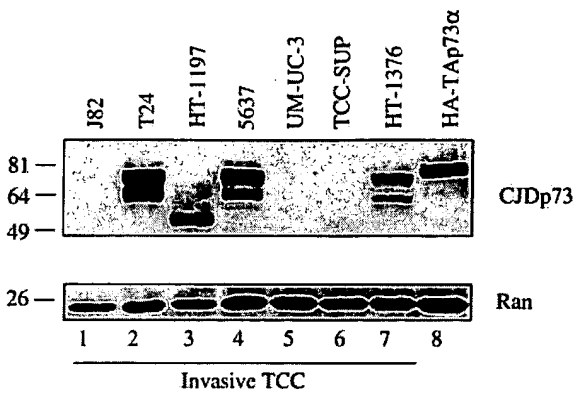

Fig. 4 p73 expression levels in bladder cancer cell lines. Protein extracts from seven different bladder tumor-derived cell lines were analyzed by Western blotting using CJDp73 and anti-Ran antibodies. *Lanes 1–7* depict levels of p73 on the following TCC lines J82, T24, HT-1197, 5637, UM-UC-3, TCC-SUP, and HT-1376. A protein extract obtained from 293 cells transiently expressing HA-tagged p73α was loaded onto *Lane 10* and was used as a positive control for CJDp73. The *bottom panel* shows the anti-Ran antibody used in blots from protein extracts at an equivalent loading through all lanes.

Expression of p73α in Other Normal Tissues. We observed intense p73α expression in spermatogonia cells of the testis (Fig. 3). p73α was generally undetectable in mesenchymal elements, including smooth and striated muscle. Additionally, p73α was undetectable in lymphocytes. Other organs such as liver, spleen, thyroid, and placenta had undetectable levels of p73α expression (Table 1).

Expression of p73 in Human Transitional Cell Carcinoma Cell Lines. We applied the CJDp73 antibody in immunoblotting and the RT-PCR conditions previously described to the characterization of p73α in a group of nine bladder tumor derived cell lines. Western blot analysis of the seven invasive bladder cell lines revealed a heterogeneous pattern of p73 expression (Fig. 4). Interestingly, only three of the invasive TCC derivatives expressed p73α. They showed two prominent species migrating at M_r ~75,000 and M_r ~65,000 (T24, 5637, and H-1376). The upper band is likely to be TAp73α isoform because the HA-tagged TAp73α ran slightly higher in the same gel (Fig. 4). We further examined the samples with other antibodies to check for the identity of the lower bands (M_r ~65,000 and Mr ~55,000). We could not find any other suggestion to match these bands with ΔNp73α or ΔNp73β (data not shown). In summary, the expression of TAp73α is lost in four of seven invasive tumor-derived cell lines (Fig. 4).

In an attempt to further clarify the identity of the p73 isoforms seen by Western blot, we performed RT-PCR for TA, ΔN, and the various COOH-terminal splice variants of *p73* on total RNA isolated from the nine bladder cancer cell lines (Fig. 5). We found that most of the nine cell lines expressed the TA, ΔN, and COOH-terminal splice variants tested (Fig. 5). TAp73 RNA was found in all of the bladder cancer cell lines but in SCaBER and UM-UC-3. We observed a correlation between high expression of *TAp73* RNA (Fig. 5) and TAp73α protein accumulation by Western blotting (Fig. 4). Additionally, whereas we detected ΔNp73 in all of the lines, we did not detect the *p73γ* splice variant and any TA RNA in the UM-UC-3 cell line, suggesting that these cells do not express any transcriptionally active isoform and only expresses some *ΔNp73* isoform different from gamma. SCaBER cells only expressed the *ΔNp73* isoforms.

Expression of p73α in Bladder Cancer: Relation to Tumor Stage and Grade. We examined 154 well-characterized primary bladder tumors compiled onto three tissue arrays. TCC arrays were analyzed by IHC using CJDp73 antibody (Fig. 6). p73α expression was reported both as continuous and categorical data (positive *versus* negative). Cores with no tumor or insufficient tumor cells were not used in the final analysis. Similar to what we observed in the invasive TCC-derived cell lines, most invasive tumors lost p73α expression. Although most superficial tumors (28 of 49; 57%) express p73α, only 22 of 105 (21%) invasive TCC lesions retain p73α expression (Fig. 6). p73α expression, as a continuous variable, was found to be statistically associated with tumor stage ($P < 0.0001$; Table 2). Using Fisher's exact test, p73α as a categorical variable was also found to be associated tumor stage ($P < 0.0001$; Table 3).

Analysis of p73 Expression and p53 Status in Bladder Cancer. p53 status was previously analyzed in these 154 tumor samples. We examined the correlation between tumor stage (superficial *versus* invasive) and the combined data referring to p53 status and p73α expression. Tumors were classified according to phenotypes into four categories (Table 4). The first category included those tumors with wild-type p53 and p73α-

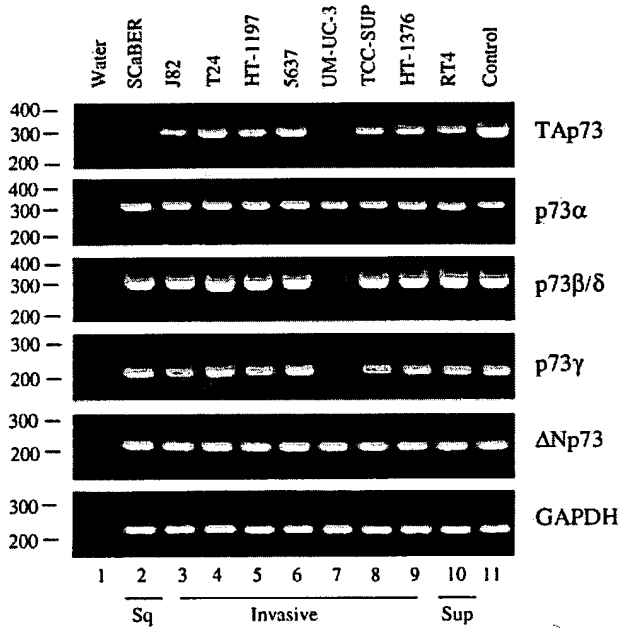

Fig. 5 Analyses of p73 transcript levels in bladder cancer cell lines. Isoform-specific sets of primers were applied by RT-PCR to a panel of nine bladder cancer cell lines. The first lane shows the water control. *Lanes 2–10* show the bladder cell lines in the following order: SCaBER, J82, T24, HT-1197, 5637, UM-UC-3, TCC-SUP, HT-1376, and RT4. The positive control is RNA obtained from a TAp73α-overexpressing cells (last lane). *GAPDH* amplification is used as a control. Most bladder cancer cells, with the exception of UM-UC-3, displayed all p73 transcripts.

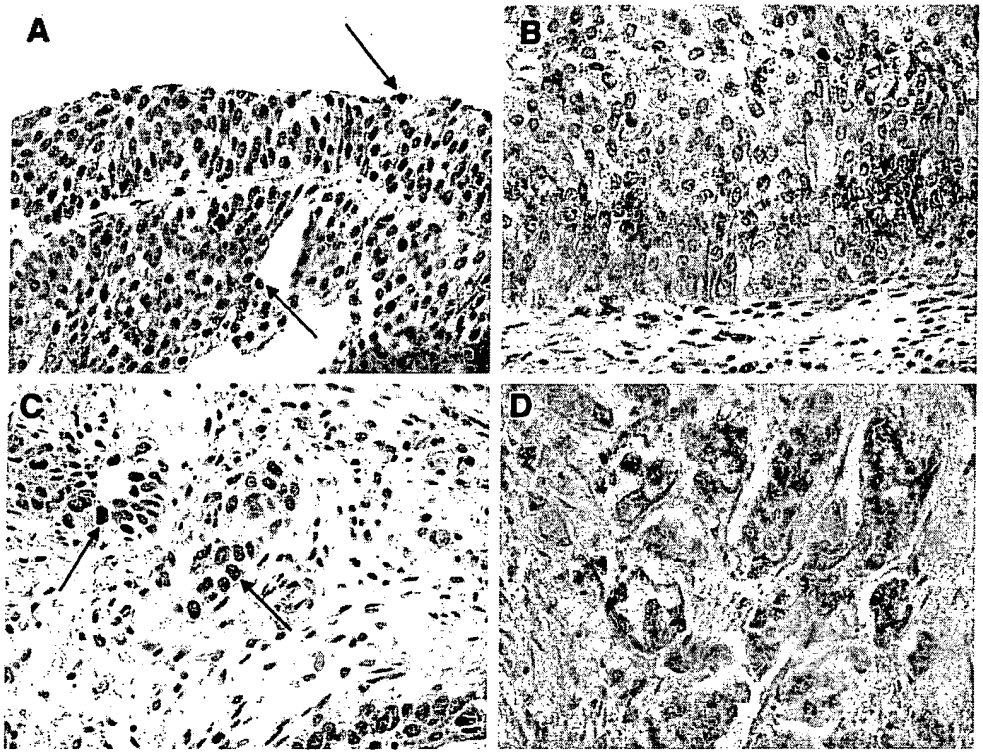

Fig. 6 Immunohistochemical analyses of p73α expression using CJDp73 antibody in bladder transitional cell carcinomas. *A* and *B* illustrate papillary superficial lesions, whereas *C* and *D* depict invasive bladder tumors. Note the intense nuclear immunoreactivities observed in certain superficial (*A*) and invasive (*C*) tumors, whereas some TCC in both superficial (*B*) and invasive (*D*) lesions had low to undetectable p73α levels. Original magnifications: *A–D*, ×200.

positive phenotype. We observed that 22 of 31 (71%) tumors in this group were papillary superficial lesions. The second category included those tumors that had wild-type p53 and a p73α-negative phenotype ($n = 62$). We observed that only 17 of 62 (27%) tumors in this group were superficial lesions. There was a significant difference between these two categories and distribution of tumor stage ($P < 0.0001$). The third category included those tumors that had a *TP53* mutation and a p73-positive phenotype ($n = 19$). We found that 6 of 19 (32%) cases were superficial lesions in this group. The final category included those tumors with *TP53* mutation and a negative p73α phenotype ($n = 42$). Only 4 of 42 (10%) tumors were found to be superficial lesions. There was a significant difference between these two last groups and tumor stage ($P = 0.06$). Furthermore, we observed a significant association between p53 status and tumor stage, independent of p73α phenotype. We observed that 39 of 93 (42%) p53 wild-type tumors were noninvasive superficial lesions, whereas only 10 of 61 (16%) p53 mutant tumors were superficial lesions ($P < 0.0001$). Interestingly, the inhibitory function of p53 over tumor progression decreases when the TCC samples have lost the expression of p73α. The group with functional p53 and a negative p73α phenotype is statistically closer to the group with mutant p53 and a negative p73α phenotype than the comparison between the groups segregated only according to p53 status ($P = 0.03$ *versus* $P < 0.0001$). p53 mutations and p73α-negative phenotype were associated with bladder cancer progression, as revealed by the comparison of this category with the groups that had a wild-type p53 and a positive p73α phenotype ($P < 0.0001$; Table 4).

Table 2 Loss of p73α expression, as a continuous variable, is associated with tumor stage

Tumor type	p73α expression[a]	Range	Cores	P^b
Invasive	4.88 ± 10.27	0–45	243	<0.0001
Superficial	17.60 ± 20.06	0–70	109	
Low grade	22.32 ± 22.11	0–70	44	<0.0001
High grade	14.99 ± 17.71	0–55	65	

[a] Mean percentage of tumor cells ± SD.
[b] F test via method of restricted maximum likelihood.

Table 3 Loss of p73α expression, as a categorical variable is associated with tumor stage

Tumor type	p73α expression: Positive	Negative	Tumors	P^a
Invasive	22 (21%)	83 (79%)	105	<0.0001
Superficial	28 (57%)	21 (43%)	49	
Low grade	10 (59%)	7 (41%)	17	1.00
High grade	18 (58%)	14 (42%)	32	

[a] Fisher's exact test.

Analysis of p73α and p63 Expression and p53 Status in Bladder Cancer. An additional level of complexity and global understanding was revealed by the study of the three family members (Table 5). Eight categories were produced based on p53 status and p63/p73α phenotypes. We would like to emphasize the significant results. The biological relevance of p63 and p73α expression is revealed by the significant association of their phenotypes with tumor stage in the back-

Table 4 Altered expression of p53 and p73α is associated with tumor stage

p53 status	p73α expression	Histology: Superficial	Histology: Invasive	Tumors	P^a	p53 status	P^a
Wild type	Positive	22	9	31	<0.0001	Wild type	<0.0001
Wild type	Negative	17	45	62			
Wild type		39	54			93	
Mutant	Positive	6	13	19	0.06	Mutant	
Mutant	Negative	4	38	42			
Mutant		10	51			61	
Total		49	105	154		154	

P^a (p53–p73)	Wild type-negative	Mutant-positive	Mutant-negative
Wild type-positive	<0.0001	0.01	<0.0001

P^a (p53–p73)	Mutant-negative
Wild type-negative	0.03

[a] Fisher's exact test.

ground of wild-type p53. More specifically, tumors that had a p53 wild-type and p63/p73α-positive phenotype ($n = 25$) comprised 21 (84%) superficial lesions, whereas wild-type p53 and p63/p73α-negative phenotype tumors ($n = 31$) included only 4 (13%) superficial lesions ($P < 0.0001$). Similarly, the cooperative effect of these three genes is revealed through the significant association to tumor stage. Tumors with a wild-type p53 and positive p63/p73α phenotype ($n = 25$) included 21 (84%) superficial lesions, whereas tumors carrying p53 mutations and negative p63/p73α phenotype ($n = 32$) included only 2 superficial lesions ($P < 0.0001$). There is no statistical difference according to tumor stage between the group with wild-type p53 and negative p63/p73α expression and the samples with mutant p53 and a p63/p73α negative phenotype ($P = 0.43$).

DISCUSSION

Using the affinity-purified anti-p73 polyclonal antiserum CJDp73 in immunohistochemical assays, we found that it detects p73α isoforms specifically, not identifying any other p73 isoforms tested. When applied to human normal adult tissues, we observed p73α immunoreactivities in basal cells lining glandular epithelia such as breast and prostate but not in the luminal cells of these organs. p73α was also expressed in basal cell layers of stratified epithelia such as the skin epidermis and in all cells of the transitional epithelium of the ureter and urinary bladder. Expression of p73α in proliferating cells such as spermatogonia and basal epidermal cells suggests a potential role for p73α in mediating tumor suppression activity, as suggested by Flores *et al.* (12). Thus, accumulation of p73α might have a

Table 5 Altered expression of the p53 family members is associated with tumor stage

p53 status	p63 expression	p73α expression	Histology: Superficial	Histology: Invasive	Tumors	P^a
Wild type	Positive	Positive	21	4	25	<0.0001
Wild type	Negative	Negative	4	27	31	
Wild type	Negative	Positive	0	5	5	
Wild type	Positive	Negative	11	18	29	
Mutant	Positive	Positive	5	5	10	0.005
Mutant	Negative	Negative	2	30	32	
Mutant	Negative	Positive	1	8	9	
Mutant	Positive	Negative	2	7	9	
			46	104	150	

P^a (p53-p63-p73)	Mutant-positive-positive	Mutant-negative-negative
Wild type-positive-positive	0.08	<0.0001

P^a (p53-p63-p73)	Mutant-negative-negative
Wild type-negative-negative	0.43

[a] Used Fisher's exact test.

preventive function in the event of cellular stress, which could menace the integrity and viability of the cell. The accumulation of p73α, as well as that reported for p63 (20), may prepare the cell to react to genotoxic insults.

The expression pattern of p73α in normal tissue is similar to that reported for p63 (28). However, there are certain differences, including the more predominant expression of p63 on the suprabasal layer of stratified epithelia when compared with the intense basal expression of p73. In addition, p63 was not detected in umbrella cells of the bladder, whereas p73α is expressed in these cells (Fig. 3). Similarly, p63 was not detected in the pancreas, whereas we report herein the expression of p73α in exocrine pancreas.

Our previous observation that normal bladder urothelium expresses high levels of p63 and that the loss of p63 expression is associated with tumor progression in bladder cancer (20) prompted us to investigate the role of p73 in transitional cell carcinoma. TAp73α expression was lost in four of seven invasive derived cell lines (Fig. 4). We also observed that p73α was more frequently lost in invasive TCC cases than in superficial TCC lesions. Statistical analyses revealed that loss of p73α expression was a frequent event associated with advanced tumor stage in bladder cancer. Our findings are in agreement with a study recently reported showing a decline in p73 expression in esophageal cancer (29).

In our study, we also compared p73α, p63, and p53 expression patterns in the same cohort of bladder cancer patients (19, 20). Although the three genes have some shared functions their expression patterns in normal tissue are different. Although p53 levels are very low, as a matter of fact undetectable by IHC, p63 and p73 are usually expressed in the nuclei of certain normal cells, including urothelium. Interestingly, it appears that the loss of p63 and p73 expression, as assessed by IHC, represents an epigenetic tumor suppressor event, because tumor-specific mutations have been reported to be very rare (see below).

p73α expression and p53 status were both significantly associated with tumor stage. We observed that the impact of p53 status in samples displaying the p73α-positive phenotype was less significant than p73α expression in samples with wild-type p53. Thus, p73α activity appears to have a critical tumor suppression function. Moreover, p73α expression and p53 status were found to have a negative cooperative effect and to be significantly associated with tumor stage.

We also observed a negative cooperative effect for the three p53 family members. Tumors with wild-type p53 and positive p63 and p73α phenotype had a significantly better prognosis than those with wild-type p53 and undetectable p63 and p73α. The dependence of p53 on p63 and p73α expression is in concordance with the results of Flores *et al.* (12). These investigators have reported that p63 and p73 expression is needed to induce a p53-dependent response to DNA damage in E1A-expressing cells and in the developing mouse central nervous system. There is a strong association between p53, p63, and p73 alterations with bladder cancer progression, as revealed by comparing the group with wild-type p53 and positive p63 and p73α phenotype *versus* the p53 mutant group lacking p63 and p73α expression with tumor stage (Table 5). The collaborative effect of p63 and p73α on the p53 function is also revealed by comparing the TCC samples with wild-type p53 *versus* mutant p53 when both groups share a negative phenotype for p63 and p73α. The dependence of the wild-type p53 function on p63 and p73α to block bladder tumor progression can be hypothesized because both groups have a highly similar fraction of invasive tumors, and the only difference is the p53 status. On the basis of data presented here, it can be postulated that assessment of p53 status alone might not be as robust as if incorporating the status of p63 and p73α.

In conclusion, this study reports the characterization of the CJDp73 antibody by Western blot and IHC on normal and tumor tissue, as well as the RT-PCR study of *p73* expression in nine bladder tumor-derived cell lines. It also reports the significant association between p73α loss and advanced tumor stage in bladder cancer. In addition, it describes for the first time the negative collaborative effect produced by alterations of the three p53 family members in bladder cancer progression using a large and well-characterized cohort of patients. Results from this study support a tumor suppressor role for p73α, where inactivation appears to be through epigenetic events in bladder cancer.

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